ON THE SPEED OF DEVELOPMENT OF INSECT EGGS AT CONSTANT TEMPERATURES

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(Accepted for publication 3rd September, 1942.)

When the reciprocals of the time required for the eggs of an insect to develop and hatch, at given temperatures, are plotted against temperature, the distribution of the points describes a sigmoid course. This occurs also with the development of the eggs of Amphibia (Moore, 1939; Ryan, 1941), and of fishes (Price, 1940). A similar distribution of the points is obtained when temperature-development data for the post-embryonic stages of insects are plotted in this way. It would appear, from the evidence available, that this trend is a fundamental feature of the relationship between temperature and rate of development in poikilothermic animals. It is also a feature of the relationship between temperature and certain other expressions of physiological activity in these animals, for instance the rate of oviposition in insects (Harries, 1939).

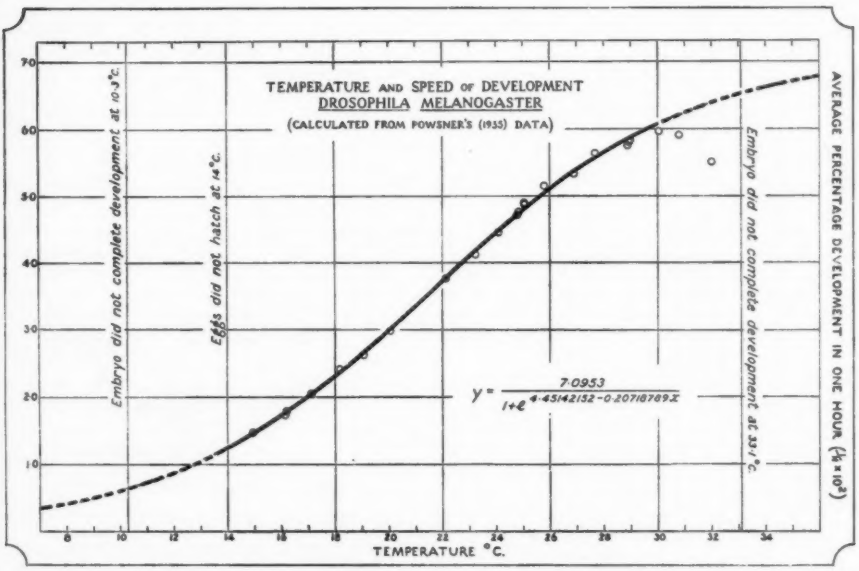

Fig. 1. Calculated curve showing the trend in the speed of development of the eggs of *Drosophila melanogaster* (Diptera) at constant temperatures.

Various formulae have been used by biologists for the purpose of expressing the relationship of temperature to the rate of development in insects: these formulae have been reviewed by several authors (Uvarov, 1931; Bělehrádek, 1935). A formula is described in the present paper which gives a satisfactory empirical representation of the trend in the speed of development in insects, as determined by temperature. Its suitability for this purpose has been demonstrated by the analysis of data, for nine species of insects, on the time taken for their eggs to

develop and hatch at constant temperatures: the data for three of the species are discussed here. The egg stage of insects was selected because accurate data from experiments are more readily available for this stage than for the active stages. It is evident from the literature, as shown by the graphs illustrating the relationship between the rate of development and temperature in *Popillia japonica* given by Ludwig (1928), that the formula can be used for the active stages of insects.

Development of the eggs of Drosophila melanogaster.

Powsner (1935) gives experimental data showing the time required for the eggs of *D. melanogaster* to develop to hatching at 22 different constant temperatures. The eggs were laid at 25° C., and then exposed at given temperatures, within half an hour of being laid. The 23 points shown in Fig. 1 represent the reciprocals of time in hours × 100 plotted against the 22 values for temperature. The "y" axis represents the average percentage development per hour attained by the eggs at each temperature. Hatching of the eggs was obtained over a range of temperature extending from 14·95° C. to 32·00° C. The speed of embryonic development, as determined by the time taken for the eggs to complete development and hatch, increased with rise in temperature until 29·5° C. was reached. Above 29·5° C. the speed of development rapidly decreased as the temperature increased: at 33° C. the eggs failed to hatch.

The 23 observed points (Fig. 1) describe an "S" shaped course which exhibits a marked change in direction about 29·5° C. At this temperature the eggs take the shortest time to develop and hatch: this implies that the embryo develops at the maximum average rate about 29·5° C. The change in the trend of the observed points at 29·5° C. suggests that changes occur in the physiology of the developing eggs, about this temperature, which are markedly different from those occurring at lower temperatures.

The 21 observed values for "y" at temperatures between 14·95° C. and 30·05° C. were fitted to a curve of the form:

$$y = \frac{K}{1 + e^{a - bx}}$$

This is the form of the Verhulst-Pearl logistic curve used by the writer to describe the trend of growth in animal populations (Davidson, 1938).

K is a calculated constant representing the upper asymptote of the curve.
y represents the average percentage development of the eggs per hour at the given constant temperatures.
x is a given constant temperature.
a is the constant which defines the relative position of the origin of the curve on the temperature axis.
b is a constant which denotes the parameter of increase in the rate of development: it determines the slope and course of the curve.

The calculated values for the above constants, computed from the data, are $K = 7{\cdot}0953$; $a = 4{\cdot}45142152$; $b = {\cdot}20718789$. Therefore the calculated formula for the curve is:

$$y = \frac{7{\cdot}0953}{1 + e^{4{\cdot}45142152 - {\cdot}20718789x}}$$

The curve shown in Fig. 1 has been calculated from this formula. The 21 observed values for "y" for the respective values of "x" between 14·95° C. to 30·05° C. are a good fit to the curve. For each of these values, the deviation from the calculated value lies within the range of ·005 to 2·15 p.c. of the calculated value. The root-mean-square deviation for the observed and calculated values is ·057957. For any given value of "y" the appropriate value for "x" can be calculated as follows:

$$x = \frac{1}{b \log.e}\left(a \log.e - \log\frac{k - y}{y}\right)$$

From the mathematical properties of the calculated curve it is seen that acceleration in the rate of development of the eggs of *D. melanogaster* increases with rise in temperature, until the temperature value at the inflexion of the curve ($x = \frac{a}{b} = 21{\cdot}5°$ C. when $y = \frac{K}{2} = 3{\cdot}55$) is reached. Beyond the inflexion the *relative* rate of development decreases with rise in temperature until about 29·5° C. is reached. At this temperature the eggs attain the maximum average speed of development (5·9 p.c.): it may be defined as the "*peak temperature*"[1]. Above the peak tem-

[1] The term "optimum" temperature is used by some authors to indicate the temperature at which the maximum rate of development of an organism takes place. The term is misleading applied in this way, and is more correctly defined as the range of temperature at which development is completed in the relatively shortest time and the greatest number of individuals survive (Janisch and Maercks, 1933; Kozhantschikow, 1936). The "optimum" temperature defined in this way, may be lower than the "peak" temperature.

perature (29·5° C.) the time required for the eggs to develop to hatching increases rapidly as the temperature is raised. There is also a marked increase in the mortality of the eggs, as determined by the smaller number which survive to hatching: for instance, at 32° C. only a small percentage of the eggs hatched; at 33·1° C. no eggs hatched. These observations suggest that the developing eggs experience harmful effects at temperatures above 29·5° C., which are intensified with increase in the temperature. The degree of harm will depend upon the temperature, duration of exposure of the eggs and stage of development of the embryo. It can be assessed by appropriate experiments in which the eggs are exposed, for different periods, to temperatures above 29·5° C., and then transferred to lower, favourable temperatures, the time required for the eggs to hatch at the latter temperatures, and the number of eggs which hatch, being recorded. The upper limit of temperature at which eggs can develop to hatching, evidently lies between 32 and 33·1° C.; it may be placed tentatively at 32·5° C. Partial development of the embryo goes on at temperatures slightly higher than 32·5° C., but the upper limit, at which all development is inhibited, was not determined.

The lowest temperature at which hatching was obtained was 14·95° C.: the eggs fail to hatch at 14° C. Therefore the zero temperature for complete development to hatching lies between 14 and 14·95° C.; it may be placed at 14·5° C. Partial development of the embryo goes on at temperatures below 14·5° C.: Pownsner states that some development occurs at 10·3° C. The lower limit of temperature at which all development is inhibited (developmental zero) was not determined. The calculated curve is a guide to the speed of development of the eggs at all temperatures between the hatching zero (14·5° C.) and the peak temperature (29·5° C.). This is 83·3 p.c. of the total range of temperature in which complete development of the eggs, as determined by hatching, takes place (14·5 to 32·5° C.).

If the eggs are maintained too long at temperatures below the hatching zero, they will die. The intensity of the harmful effects will depend upon the temperature, period of exposure of the eggs and the stage of development of the embryo. It could be assessed by approximate experiments in which the eggs are placed at favourable temperatures after exposure to temperatures below the hatching zero, the time required for the eggs to complete development and hatch at these temperatures, and the number of eggs which hatch, being recorded. The actual developmental zero temperature might be determined by examination of stained sections of eggs which had been exposed for suitable periods at appropriate low temperatures, to see whether any embryonic development had taken place. Appropriate temperatures can be selected by reference to the calculated curve in Fig. 1. For example, at 8° C. the embryo might be expected to make an average of ·4 p.c. of its development per hour, if the predicted rate follows the calculated curve, and if this temperature is not below the developmental zero. It should be noted however, that, at the same temperature, the rate of development of the embryo in its early stages of development may not be the same is in the later stages.

Development of the eggs of Habrobracon juglandis *Ashm.*

The plotted data, shown in Fig. 2 as small circles, represent the reciprocal values × 100 for the time taken for the eggs of *H. juglandis* to develop and hatch at the given constant temperatures. The values were calculated from data obtained by Maercks (1933). The insects were reared at 22° C., and the eggs were placed at the given temperatures within 20 minutes of being laid. The lowest temperature at which the eggs could develop to hatching was 13·4° C., the time required being 201 hours. Partial development of the embryo was observed at 11·9° C., but development could not be completed at this temperature: the actual developmental zero was not determined.

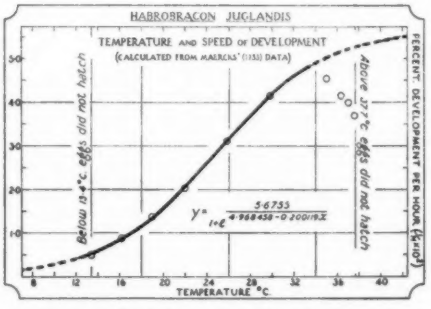
HABROBRACON JUGLANDIS
TEMPERATURE AND SPEED OF DEVELOPMENT
(CALCULATED FROM MAERCKS' (1933) DATA)
Below 13·4°C. eggs did not hatch
Above 37·7°C. eggs did not hatch
PERCENT. DEVELOPMENT PER HOUR (1/HRS)
TEMPERATURE °C

Fig. 2. Calculated curve showing the trend in the speed of development of the eggs of *Habrobracon juglandis* (Hymenoptera) at constant temperatures.

The upper limit of temperature at which the eggs could develop to hatching was 37·7° C., the time required being 27 to 33 hours. Partial development of the embryo was observed at 38° C., but development could not be completed at this temperature. The upper limit of temperature, at which development was entirely inhibited, was not determined.

The shortest time required for the eggs to complete development to hatching is given by Maercks at 21 hours at 33° C. The six observed values for "y" at the six temperatures extending from 13·4° C. to 33° C. (Fig. 2) have been fitted to a curve whose formula is:

$$y = \frac{5 \cdot 6755}{1 + e^{4 \cdot 968458 - \cdot 200119x}}$$

The curve shown in Fig. 2 has been calculated from this formula. The six observed values for "y" are a good fit to the curve. For each of these values the deviation from the calculated value lies within the range of ·144 and 4·971 p.c. of the calculated value. The root-mean-square deviation of the observed and calculated values is ·02748.

The observed points depart somewhat abruptly from the calculated curve when the temperatures exceed 33·5° C. This may be considered tentatively as the "*peak temperature*". The computed values for "y" at this temperature, calculated from the above formula, is 4·82 p.c.: the shortest time observed by Maercks, namely 21 hours at 33° C., represents an observed value y = 4·76 p.c. when x = 33° C. The number of eggs which hatched decreased rapidly as the temperature was raised from 33·5° C. to 37·7° C.: there was also a marked increase in the amount of variation in the hatching time, at these high temperatures. The lowest mortality of the eggs, and the smallest variation in the hatching time, was obtained between 29° C. and 30° C., which represents the "optimum" range of temperature as defined by Janisch and his associates.

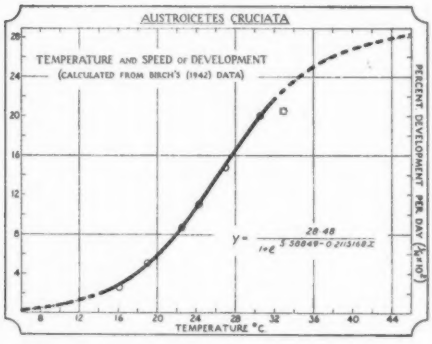

Fig. 3. Calculated curve showing the trend in the speed of the development of post-diapause eggs of *Austroicetes cruciata* (Orthoptera) at constant temperatures.

The calculated curve is a guide to the average speed of development of the eggs at temperatures between 13·4 to 33·5° C., that is for 82·7 p.c. of the total range of temperature in which complete development to hatching takes place (13·4 to 37·7° C.).

Development of the Post-diapause Eggs of Austroicetes cruciata *Sauss.*

The speed of development of post-diapause eggs of *A. cruciata*, at seven constant temperatures, is shown in Fig. 3. The plotted points represent the average percentage development per day (y) which takes place at the given temperatures (x): the values are taken from data obtained by Birch (1942). The grasshopper *A. cruciata* lays its eggs in November: the embryo partially develops and then passes into a state of diapause, which in nature continues during the following six months. When eggs in a state of diapause are exposed to temperatures which are favourable for the development of post-diapause eggs, they do not develop at the normal rate: when the diapause state is ended, subsequent development of the embryo proceeds in the normal way at these temperatures (Birch, 1942).

Data for the time required for post-diapause eggs to complete their development and hatch were obtained for temperatures ranging from 16·1 to 32·9° C. At 13·5° C. only one egg hatched out of 150 eggs: it hatched on the 138th day: when the remaining eggs were transferred to 25° C., many of the eggs hatched within a few hours of being placed at 25° C. It is seen from the calculated curve in Fig. 3, that the expected speed of development at 13·5° C. is 1·6 p.c. per day. If this extrapolated value is true, this implies that the embryo could have completed its development about the 62nd day, but it remained in the egg case until the 138th day. Evidently the embryo can complete its development at 13·5° C., but the nymph is not adequately stimulated to emerge from the egg at this low temperature. The evidence suggests that the zero temperature for hatching may be about 14° C. Although some movement of the embryo was observed at 8° C., it is doubtful whether this movement can be interpreted as development: the actual developmental zero was not determined.

The highest temperature at which complete development of the eggs to hatching was obtained was 32·9° C.: the upper temperature limit, at which all development is inhibited, was not determined; it may be placed tentatively as 34° C.

The observed values for "y", for the six temperatures extending from 16·1 to 30·5° C., were fitted to a curve having the formula:

$$y = \frac{28 \cdot 48}{1 + e^{5 \cdot 58849 - \cdot 211517x}}$$

The curve shown in Fig. 3 was calculated from this formula. The six observed values for "y" are a good fit to the curve. For each of these values the deviation from the calculated value lies within the range of ·36 and 7·47 p.c. of the calculated value. The root-mean-square deviation for the observed and calculated values is ·23833.

From the position of the observed point $y = 20 \cdot 42$ when $x = 32 \cdot 9$, it will be expected that the observed values will begin to turn away from the calculated curve at a temperature value somewhere between 30·5° C. and 32·9° C. The temperature at which the post-diapause eggs make their maximum average speed of development *(peak temperature)*, was not determined; it may be placed tentatively at 32° C.: the calculated average speed of development at this temperature is 21·5 p.c. per day.

Above the "peak" temperature, the speed of development rapidly decreases with increase in temperature, until the upper temperature limit for hatching is reached. The calculated curve is a guide to the average speed of development of the post-diapause eggs at temperatures extending from the hatching zero (14° C.) to the "peak" temperature (32° C.), which is 90 p.c. of the total range of temperature (14 to 34° C.) in which it is considered that complete development to hatching can take place.

DISCUSSION.

The formula which is described in this paper can be used to calculate the rate of development of the eggs of insects for temperatures extending from the hatching zero to the *peak temperature*. A formula which is widely used by entomologists for calculating the rate of development of an insect at given temperatures, is based on the theory that every stage in the life cycle requires a definite amount of heat for its development. The amount of heat is defined as the thermal constant, which is expressed in degree units, such as day-degrees or hour-degrees. The theory is based on the work of various authors, notably Sanderson and Peairs, Krogh, Blunck and Bodenheimer (Uvarov, 1931). According to the theory, if the reciprocals of the time required for development (y) are plotted against temperature (x) the points lie on a straight line. The line represents the temperature-velocity curve: when it is projected to cut the temperature axis, it does so at a value "c" which is defined as the theoretical developmental zero temperature. It is generally known that the reciprocal values approximate to a straight line, only over a restricted, median portion of the total range of temperature at which development goes on. This is shown in Fig. 1. Pownsner states that the straight-line temperature-velocity curve, obtained by the above method, cuts the temperature axis at 12° C.: this temperature, therefore, represents the theoretical developmental zero for the eggs of *D. melanogaster*. It is seen in Fig. 1 that only a few of the observed points, situated about the inflexion of the calculated curve ($x = 21 \cdot 5$° C., $y = 3 \cdot 55$), can be considered as approximating to this straight line. Since the trend of the reciprocal values follows a sigmoid course, it is evident that the proposed straight-line velocity curve is determined by an arbitrary selection of points in the median temperature range. Therefore, the theoretical developmental zero, obtained in this way, is an unreal, arbitrary value. If the straight-line velocity curve is used to calculate the time required for the particular stage of any insect concerned to complete its development, the computations must be interpreted with great caution, due regard being given to the actual median range of temperature, in which the observed reciprocal values fit the selected straight line: computed values for temperatures outside this range will differ greatly from the observed values. It may be noted, if a straight line is drawn in Fig. 1 through the value for "y" at the peak temperature (29·5° C.) and at 21·5° C. (temperature at the inflexion of the curve), the line cuts the temperature axis at 9·5° C. Since the two halves of the calculated curve are symmetrical about the point of inflexion ($y = 3 \cdot 55$ when $x = 21 \cdot 5$° C.), the value of 9·5° C. is a useful one, which is fixed by two defined points on the curve: in this instance it gives a closer approximation to the zero-temperature for development than the value 12° C. given by Powsner.

Janisch in 1925, and in subsequent papers, maintains that the relationship between temperature and the time required for development in insects can be expressed by a catenary curve, which fits observed data throughout the whole range of temperature at which development can take place. The formula of the curve is:

$$y = \frac{M}{2}(C^x + C^{-x})$$

When the constant "C" is the same value in both cases, the curve is symmetrical, and "M" represents the shortest possible time that the stage of development concerned can be completed. When the constant "C" has one value in the positive exponential and another value in the negative exponential, the catenary curve is asymmetrical, and the value for M does not coincide with the minimum time for development. "y" represents the time required for development at the given temperature "x".

The formula implies that the effect of the combination of factors which determine the trend in the rate of development of an organism in relation to temperature is expressed by the sum of the two exponential curves C^x and C^{-x}. At temperatures below the "peak temperature", the influence of the exponential C^{-x} increases as the temperature falls, while the influence of the exponential C^x decreases: at low temperatures the influence of C^{-x} is very large, and C^x very small. At temperatures above the "peak temperatures" the influence of the exponential C^{-x} diminishes as the temperature rises, while the influence of C^x increases: at high temperatures the influence of C^x is very large and C^{-x} very small. The use of the catenary curve, as employed by Janisch, has been adversely criticized by some authors, notably by Martini (1928). It is a general type of curve which was based on poor data.

In Fig. 1 there is an insufficient number of observed points about the peak temperature, and the temperatures immediately above it, to show whether the trend of the rate of development follows the smooth course as defined by the catenary curve. Vouté (1936) considers that the observed points do not do so in the case of *Ephestia kuhniella*. We have seen that there is evidence of the harmful effects of heat on the organism at temperatures above the peak. These effects are intensified as the temperature increases and the period of exposure is extended. Therefore, observed data for rate of development at temperatures above the peak temperature, will be less reliable than data for temperatures below the peak. Moreover in Fig. 1 the range of temperature, beyond the peak, at which the eggs can develop to hatching, is only 16·7 p.c. of the total temperature range in which development to hatching can take place. It is not possible to calculate the formula which represents the trend of the observed points, above the peak temperature, from data covering such a limited range of the predicted curve. It is undesirable, owing to our lack of knowledge about the changes which take place in the physiology of the organism at temperatures above the peak, to assume that the observed points follow the particular trend implied by the catenary curve. For these reasons it seems to the writer that it is best to use only the observed data for temperatures at, and below, the peak temperature, when fitting the appropriate curve. It should be noted that value for the peak temperature must be carefully determined by experiment. The value is required in the calculation of the constant K in the formula described in this paper: it is the guide to the direction of the upper part of the calculated curve. If it is necessary to find out the approximate period for development at temperatures above the peak, the observed points can be joined by a freehand curve, from which the required values for y at given values for x, can be read off.

Acknowledgments. I am indebted to my colleagues in the Department of Entomology for helpful discussions on the subject of this paper, and to Miss N. M. Brookes for making the finished drawings for the figures.

SUMMARY.

A study has been made of time-temperature data relating to the rate of development of the eggs of three species of insects, with the object of illustrating the trend in the speed of development in poikilothermic animals, as determined by temperature.

The observed data covering the whole range of temperature at which development to hatching takes place, can be divided into two groups, namely: (1) data

extending from the lowest temperature to the temperature at which development proceeds at the fastest rate, defined as the *peak temperature*, which covers 82·7 to 90 p.c. of the total temperature range: (2) data extending from the peak temperature to the highest temperature at which development to hatching can go on: this covers the remaining 17·3 to 10 p.c. of the total temperature range.

An exponential curve of the form: $y = \frac{K}{1 + e^{a-bx}}$ faithfully represents the trend in the speed of development over the temperature range included in group (1). The straight-line velocity curve of various authors is briefly discussed: also the catenary curve of Janisch (1925).

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SOME EFFECTS OF REMOVING THE NUCLEUS FROM AMOEBA[1]

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(Accepted for publication, 28th October, 1942.)

This paper is a brief report of some aspects of work carried out during the last two years on the physiological functions of the cell nucleus. Owing to war conditions, the report has been made as concise as possible and only the most essential details of both results and experimental procedure have been given. Reviews of earlier work on the subject are contained in the papers of Willis (1916), Lynch (1919) and Becker (1926).

Amoeba proteus has been cultivated in pure lines in this laboratory for some years past and individuals taken from these cultures were used throughout the work. Enucleated amoebae were obtained in one of two ways: (i) by cutting normal amoebae in halves under a binocular microscope, using a fine glass thread, and (ii) be removing the nucleus directly with microneedles and a micropipette operated by means of a Zeiss micromanipulator. Removing the nucleus directly has the advantage that hardly any cytoplasm is removed at the same time; the bulk of the amoeba remains practically unchanged. With practice these procedures do not injure the amoeba in any other way, and the enucleated individuals live for days.

As soon as an amoeba has been deprived of its nucleus, there is a gradual decrease in streaming activity, followed by the withdrawal of pseudopodia and the loss of attachment to the substrate. Within ten or fifteen minutes the protoplasm has rounded itself off and passes into a condition which, for want of a better name, is referred to as corrugation, the plasmalemma showing a large number of blister-like processes. On the following grounds, it is believed that this condition develops owing to excessive loss of water following the mechanical stimulus of enucleation. A similar condition can be induced in a nucleated amoeba by placing it for 15 minutes in a 20 p.c. sugar solution. Furthermore, during the corrugated condition, no contractile vacuole can be detected. It seems that with the great loss of water over the whole surface of the amoeba there is no necessity for the vacuole to perform its osmoregulatory function. Finally, if the enucleated amoeba is placed in doubly distilled water immediately after the operation, no corrugated condition develops. As the saline concentration of the internal fluid of an amoeba is about 0·005 M (Mast and Fowler, 1935), there would be a considerable osmotic flow of water into the amoeba under these conditions tending to balance any water loss due to stimulation. The corrugated condition lasts for about half an hour but is greatly prolonged by rough handling of the amoeba during enucleation. After recovering from this condition, the amoeba shows varying degrees of activity. Locomotion is always slow and of the limax type; it is highly irregular in rate and direction; there is seldom firm attachment to the substrate and it is always accompanied by an extreme degree of solation of the cytoplasm, i.e. the gel/sol ratio is abnormally low. For information on the ratio of plasmagel and plasmasol and locomotion in nucleated amoebae, reference should be made to papers by Mast (1926) and Mast and Prosser (1932).

Locomotion, whether rapid or slow, does not last for any great length of time; the amoeba gradually passes into a quiescent condition, becoming more or less spherical in shape. During the remainder of its survival the enucleated cytoplasm passes through alternating periods of activity and quiescence but, as the latter condition is always of greater duration, casual observation usually reveals an inactive amoeba. However, continuous observation reveals the frequent return of periods of activity lasting for a few minutes to as much as two to three hours. This behaviour persists until the death of the amoeba, which occurs usually within 8 days. The alternating phases of activity and inactivity are difficult to explain. How is it that the absence of a nucleus prevents an amoeba from being active on one day but not on the next? As the amoebae were always kept under conditions of starvation food does not appear to be a factor. When nucleated and enucleated amoebae are kept under identical conditions of starvation, the nucleated individuals outlive the enucleated ones. This is indicated in Table 1. Twenty amoebae were starved for four days until all food vacuoles were eliminated. They were then cut into approxi-

[1] This work, suggested and directed by Prof. W. E. Agar, F.R.S., was carried out during the tenure of a Howitt Research Scholarship.

mately equal halves and were placed in distilled water without any food. Each day the number of nucleated and enucleated individuals still alive was determined.

TABLE 1.

Days.	Number found dead, nucleate.	Number found dead, enucleate.
1	–	–
2	–	–
3	–	–
4	–	1
5	–	2
6	2	3
7	2	4
8	–	5
9	1	3
10	2	1
11	2	–
12	3	1
13	3	
14	2	
15	1	
16	1	
17	1	
	Mean day of death 11·4	7·4 days

If inability to use food were the cause of the peculiar behaviour and eventual death of an enucleate amoeba, it would seem natural to expect that by placing nucleated and enucleated individuals under comparable conditions of starvation they would live for the same length of time and would behave similarly. Table 1 does not support this supposition, nor does the observation that during the period of starvation, the locomotion and behaviour of the nucleated amoeba are quite normal. Possibly during locomotion, end-products are produced which, in the absence of the nucleus, are not oxidized and result in an autointoxication persisting until the metabolites have diffused away. If this is so, washing an enucleated amoeba should render it more active by aiding in the removal of these substances. This has been found to be the case. Frequent washing of enucleated amoebae by transferring them every hour or so into fresh amounts of medium definitely increases their activity, although they do not seem to show any improvement in the powers of attaching themselves to the substrate. Consequently locomotion is still very slow.

If inability to oxidize end-products is characteristic of an enucleated amoeba, one might expect the oxygen consumption of the cell to be very low during the entire survival period. Increased activity due to the removal of metabolites by diffusion would not involve increased oxidations. On the other hand, with no accumulation of metabolites, increased activity would be accompanied by a corresponding increase in oxygen consumption, the increment being due to the oxidation of metabolites resulting from locomotion. These considerations led to attempts being made to measure the oxygen consumption of a single amoeba, when nucleated and enucleated.

The apparatus used for the purpose was the capillary respirometer of Howland and Bernstein (1931), whose paper should be consulted for complete technical details. The amoeba is drawn into a capillary tube of about 0·3 mm. internal diameter, together with an air bubble which separates the medium from a droplet of 0·5 p.c. potassium hydroxide solution. The ends of the tube are sealed with paraffin oil. Although the tubes can be filled by hand, the operation is made much easier by using a Chamber's micro-injection system. The alterations in the length of the air-column are measured at intervals of half an hour and, knowing the diameter of the tube, it is possible to convert changes in length into changes in volume. To eliminate local changes in the oxygen content of the medium, an individual *Stylonychia* was at first introduced into both experimental and control tubes, its active movements having a stirring action. However, after several trials had been made, it appeared doubtful whether it was of any real value, and in all later

experiments the ciliate was omitted from the tubes. During the experiments, the respirometer tubes were placed in a Caneite box and were left in subdued light in the cool room; the temperature remained fairly constant throughout the experiment. To facilitate observation and measurement under the microscope, the tubes were placed on a standard microscope slide and were covered with a drop of oil and a cover slip. In this manner, the amoeba could be examined with a $\frac{1}{4}$" objective at any time during the experiment. Linear relations were obtained in all cases for oxygen-consumption/time graphs. The amount of oxygen consumed per hour was found to be proportional to the size of the amoeba. Consequently, all the enucleated amoebae used in this series of experiments had the nucleus removed directly with the micromanipulator, in order that the bulk of the cytoplasm should be as large as possible.

TABLE 2.

No. of experiment.	Oxygen consumed per hour.	Difference from mean.
1	$1{\cdot}6 \times 10^{-3}$ mm.^{3}	0·2
2	1·2	— 0·2
3	1·3	— 0·1
4	1·8	0·4
5	1·5	0·1
6	1·3	— 0·1
7	1·4	0·0
8	1·2	— 0·2
9	1·4	0·0
10	1·3	— 0·1

Mean: $1{\cdot}4 \times 10^{-3}$ mm.^{3} per hour. Standard Dev. 0·03.

TABLE 3.

Protozoan.	Oxygen consumption at room temperature.	
Paramoecium caudatum	$5{\cdot}2 \times 10^{-3}$ mm.^{3} per hr.	Kalmus (1928)
Spirostomum sp.	2·5 " " " "	Specht (1934)
Actinosphaerium sp.	1·13 " " " "	Howland (1931)
Stylonychia sp.	2·34 " " " "	Clark (Unpubl.)

The mean rate of oxygen consumption of an intact, nucleated amoeba, calculated from the figures in Table 2 is $1{\cdot}4 \times 10^{-3}$ mm.^{3} per hour. This figure is of the same order of magnitude as those obtained for other protozoa (see Table 3). Following enucleation there is at first an initial rise in the rate of oxygen consumption, but after 8 to 10 hours the rate falls to a very low value, rather less than $0{\cdot}2 \times 10^{-3}$ mm.^{3} per hour. Such extremely small volumes as this are very close to the limits of sensitivity of the apparatus employed. Figures from a typical experiment are shown graphically in Fig. 1.

The very small oxygen consumption shown by an enucleated amoeba some hours after enucleation appeared to be due to some failure in the respiratory mechanism. The nature of this mechanism was examined by determining its degree of sensitivity to dilute potassium cyanide. Nucleated and enucleated amoebae were immersed in M/1000 KCN solution and were placed in the respirometer tubes in that solution. The mean results of several experiments are given in Table 4.

TABLE 4.

Amoeba.	Oxygen consumption in mm.^{3} per hour.	No. used.
Normal nucleated	$1{\cdot}4 \times 10^{-3}$	10
Enucleated	0·2 "	10
Normal nucleated in KCN	0·3 "	5
Enucleated in KCN	0·2 "	5

These figures show that enucleation and immersion in dilute potassium cyanide both reduce the oxygen consumption of an amoeba by about 70 p.c. It must be regarded as being highly significant that the already low oxygen consumption of an enucleated amoeba does not appear to be further depressed by cyanide. In other words, the respiration of an enucleated amoeba is of the residual type, being insensitive to cyanide. It would seem that the oxygen-carriers concerned with 70 p.c. of the normal respiration of an amoeba are related in some way to the presence of the nucleus. In this connection it is interesting to note that it is possible to demonstrate the presence of organic iron-containing compounds in the nucleus of

amoeba by means of a modified Turnbull blue reaction. It is hoped that it will prove possible to give a more complete account of certain aspects of this work at a later date. Here only the more important of the results are considered.

During the periods of activity, it is possible that the oxygen consumption of the enucleated amoeba returns to a more nearly normal value. There has been no indication of this in any of my experiments, but the matter cannot be regarded as settled for in most cases the active periods are of such short duration that it is difficult to get an enucleated amoeba to remain active inside the respirometer tube long enough for a significant measurement to be made. Attempts to measure the oxygen consumption of an amoeba over a period of several days have failed, owing to gradual diffusion of the CO_2 absorbent into the medium containing the amoeba. No success has been met with in devising other types of respirometer sensitive enough to register the minute amounts of oxygen concerned.

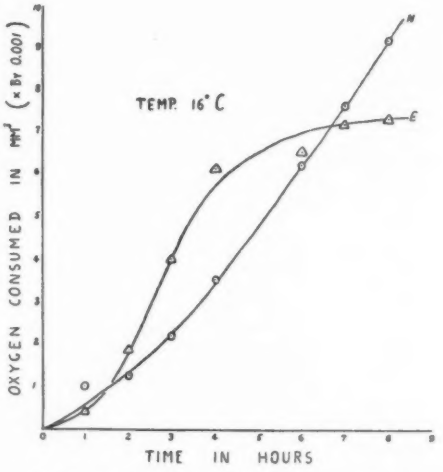

Fig. 1. Graph showing the oxygen consumption of a nucleated (circles) and an enucleated (triangles) amoeba. Note the marked fall occurring about five hours after enucleation.

Lynch (1919) reaches the rather strange conclusion that the metabolic rate of an enucleated amoeba, as measured by Child's method, is higher than that of a nucleated amoeba, although at the same time he admits that enucleated amoebae are very inactive compared to normal individuals. His conclusion is not in agreement with the experiments on oxygen consumption which have just been mentioned. Nor have I been able to repeat Lynch's experiments with the same results. Briefly, he estimated the metabolic rate by immersing the amoeba in a strong solution of potassium cyanide, in this case M/50, and measuring with a stop watch the time in seconds required for cytolysis. This time is taken as an inverse measure of the metabolic rate and according to the supporters of Child's theory of axial gradients, this metabolic rate is one of respiration. As there is still doubt as to the validity of this belief, instead of metabolic rate I have used the term cyanide sensitivity, which makes no assumption as to the underlying mechanism of the action.

Three groups of enucleated amoebae were placed in the cyanide solution at different times after enucleation. Those placed in the solution immediately after enucleation showed the same degree of sensitivity as normal control amoebae. On the other hand, those used 24 or 72 hours after the operation could be placed in one of three well-defined groups. Group I contained amoebae which were cytolysed within 90 seconds; these had been very active before being placed in the cyanide solution. Those in Group II were cytolysed within 160 seconds and, although not active immediately before being used, they had recently shown limax streaming. Group III amoebae were not cytolysed after 600 seconds and were all inactive and contracted into a more or less spherical shape before being placed in the cyanide solution.

We can thus draw the very interesting conclusion that the sensitivity of an enucleated amoeba to immersion in a strong cyanide is in some way related to the degree of recovery shown by the amoeba. A very active amoeba is highly sensitive, an inactive amoeba comparatively insensitive. It does not necessarily follow that active enucleated amoebae have a high metabolic rate for it is doubtful whether immersion of a cell in strong solution of potassium cyanide actually gives any indication of the rate of metabolism. A true cyanide inhibition of respiration is obtained with very low concentration of that substance and is not accompanied by rapid cytolysis. Consequently an attempt was made to investigate the mode of action of strong cyanide solutions in bringing about cytolysis.

When examining the distribution curves obtained during a statistical examination of the times taken for the cytolysis of normal amoebae taken directly from the cultures, it became

apparent that there was some relation between the time for cytolysis to occur, the number of pseudopodia and the degree of attachment shown by the amoeba in question. The distribution curve for amoebae taken at random from the culture was bimodal, a point of some importance, for all the amoebae were from pure line cultures and hence the differences in the times taken for cytolysis must have been due to temporary physiological differences, such as number of pseudopodia and the degree of attachment. To clear up this point, a statistical examination of the cyanide sensitivities of pure line amoebae was made upon amoebae which had been rendered as physiologically identical as possible. By washing amoebae in two changes of distilled water over a period of half an hour, individuals are obtained which show no attachment whatever but simply drift around in the water and extend from three to many very long, thin pseudopodia. Amongst workers on amoebae, this condition is referred to as the stellate condition (the "Reizzustand" of German workers).

The distribution curve obtained from this study showed only one mode, while the actual time taken for any individual amoeba to cytolyse was found to be related to the number of thin pseudopodia present. Those with many pseudopodia cytolysed rapidly, while those with only three or four pseudopodia lasted for a much longer period.

It was also found that the stellate amoebae as a class were more sensitive to the cyanide than individuals taken at randon from the cultures. As there is no ground to suspect the existence of a difference of respiratory rate in the amoebae, it would seem that the time taken for cytolysis was related to the physical condition of the amoeba and especially of the plasmalemma. This belief was supported by the finding that the cytolysis was due, not to any specific action on the part of the cyanide radicle, but to the action of the high concentration of potassium ions acting in a strongly alkaline medium. This point was demonstrated in the following manner.

The cytolysing effect of a strong solution of potassium cyanide can be reproduced by using instead a dilute solution of sodium hydroxide of pH 12. Below pH 10 no cytolysis could be produced, while even when the pH is 11, cytolysis is slow, taking as long as an hour to occur. Above pH 11·5 cytolysis is very rapid. Now the cyanide solutions used never showed a greater degree of alkalinity than pH 11 and during the majority of the experiments it was below 10·6. In other words, it does not seem likely that the alkalinity of the cyanide solution is the sole factor in producing cytolysis. It was also shown that amoebae could be immersed in very strong solutions of potassium salts showing no hydrolysis without cytolysis occurring. Hence if the cytolytic action is due to the high concentration of potassium ions, it must be due to their action in an alkaline medium. Immersion of nucleated amoebae in an M/25 solution of potassium chloride which had been made alkaline by adding KOH to pH 12 resulted in cytolysis occurring in a mean time of 21 seconds. In the absence of a high concentration of potassium ions, cytolysis in a solution of the same alkalinity occurred in a mean time of 68 seconds. Apparently the potassium ions, together with a high concentration of hydroxyl ions are much more effective in producing cytolysis, than is either factor alone. Furthermore, an M/25 solution of potassium chloride will produce cytolysis even if the pH is lowered to 10, a degree of alkalinity frequently reached by strong cyanide solutions.

The converse experiment was also made, the pH of an M/25 solution of cyanide being regulated to 7·5 by the use of an M/50 phosphate buffer. In this solution, ten amoebae lived for more than two hours without showing any cytolysis. If the pH had not been regulated, all would have disintegrated within half a minute. Finally, to make the matter quite definite, the action of the potassium ions in the cyanide solution was antagonized by adding magnesium sulphate in a concentration of M/100. For an account of antagonism in amoeba, reference should be made to the paper of Reznikoff (1927). In this cyanide solution, although the pH was higher than 10·5, no cytolysis occurred within four hours.

It is held that these experiments show conclusively that the rapid cytolysis produced by immersing amoebae in strong cyanide solutions is due to the action of the high concentration of potassium ions acting in a strongly alkaline medium. It is felt that Lynch's (1919) conclusion, based on experiments on the cytolysis of amoebae in strong cyanide, that an enucleated amoeba has a higher metabolic rate than a nucleated one, is incorrect. It is interesting to note that workers on metabolic gradients have used other toxic substances to produce cytolysis, substances which have no effect on respiration. Furthermore, most of the effects of the strong cyanide solution on amoebae, whether nucleated or not, can be reproduced to a large extent by other toxic substances such as trypaflavine or sodium alizarin sulphonate. An account of some experiments with these substances may be published at a later date. However, enough has been said to indicate that any increased cyanide sensitivity shown by a recovering enucleated amoeba is probably related to the condition of the plasmalemma during locomotion and attachment rather than to any alteration in metabolic rate. Brinley (1928) in a very interesting

paper has shown that the effects of cyanide upon amoeba are primarily due to the action of the substance on the plasma membrane and not on the internal cytoplasm. This finding is in accord with the present author's views already given.

A very interesting question is why the enucleated amoebae die. An enucleate piece of cytoplasm does not survive for more than ten days at the most without special treatment. Why is this? We have evidence to show that the respiration of an enucleated amoeba is depressed by about 70 p.c. below the normal figure. Is this a clue to the cause of death?

Experiments were made to measure the oxygen consumption of enucleated amoebae that were supplied with traces of methylene blue (1/20,000) and glucose (0·1 p.c.). The oxygen consumption was raised by varying amounts, usually to about 50–60 p.c. of its normal value but there was never any appreciable increase in the activity nor in the length of life of the enucleated amoeba. In one experiment, the oxygen consumption of the enucleated amoeba in culture water was found to be $0{\cdot}3 \times 10^{-3}$ mm.^{3} per hour. The same amoeba, when immersed in methylene blue and glucose, consumed $0{\cdot}8 \times 10^{-3}$ mm.^{3} per hour. This experiment finds its counterpart in that of Harrop and Barron (1928), who found that the low respiratory rate of non-nucleated mammalian erythrocytes can be greatly increased by suspending the cells in a medium containing methylene blue and glucose.

As the nuclei of most cells appear to contain comparatively large amounts of calcium, enucleated amoebae were supplied with this element in a soluble form. They were placed on alternate days in a solution of calcium chloride, M/500. There was always a definite increase in the activity of the amoeba and in the length of life. By this treatment it was possible to keep one enucleate amoeba alive for nineteen days. However, even under these conditions the mean length of life of the enucleated amoebae was always less than that of the nucleated ones under similar conditions.

For many years it has been established that an enucleated amoeba, although capable of ingesting food, never digests it. There does not appear to be any secretion of digestive enzymes in the absence of the nucleus. However, this is not the cause of death, for in the experiment mentioned at the beginning of this paper, it was found that when nucleated and enucleated amoebae are starved together the nucleated ones outlive the enucleate. It is the belief of the author that the cause of ultimate death is due to a dedifferentiation of the ultrastructures normally present in the cytoplasm, and maintained there by nuclear action. Many cases can be quoted to demonstrate the dependence of cytoplasmic differentiation upon the activities of the interkinetic nucleus, and it is interesting to note that a normal, nucleated amoeba undergoing mitosis shows several of the features of enucleated amoebae, such as loss of attachment, the development of the corrugated appearance and the temporary disappearance of the contractile vacuole. Many highly specialized cells undergo a complete dedifferentiation before mitotic division occurs and as it is a general belief amongst cytologists that the nucleus is in its most active condition during interkinesis, it seems possible that the differentiation is maintained by nuclear action. If this is correct, then the removal of the nucleus from an amoeba would result in dedifferentiation and a consequent inability for the cytoplasm to carry on in the normal manner. This of course introduces the concept that a differentiated condition is in the nature of a dynamic condition and not a static one and to the author, this seems to be what one would expect to be the case in a biological system.

SUMMARY.

An amoeba lives for about seven days after it has been deprived of its nucleus.

Enucleation results in a 70 p.c. depression of respiration.

About 70 p.c. of the respiration of a normal amoeba is cyanide-sensitive. The decreased oxygen consumption remaining after enucleation is not effected by cyanide.

The length of life is increased slightly by supplying small amounts of calcium chloride.

The ultimate death of an enucleated amoeba is not due to starvation nor to reduced oxygen consumption.

It is suggested that cytoplasmic dedifferentiation is the cause of death in an enucleated amoeba.

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STUDIES ON THE NITROGEN METABOLISM OF PLANTS

V. THE RELATION OF CARBOHYDRATE CONTENT TO PROTEIN SYNTHESIS IN LEAVES[1]

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(Accepted for publication, 30th July, 1942.)

INTRODUCTION.

In the literature of nitrogen metabolism in plants it is widely held that protein synthesis is dependent on carbohydrate content. Reviews of previous work have been given by Paech (1934), Kabos (1936) and Nightingale (1937).

Data contained in the foregoing which are pertinent to the work described here are as follows: Diurnal changes in amount of protein and protein changes during the life cycle of leaves attached to the plant are generally correlated with changes in the same direction of amount of carbohydrate. Formation of new leaves and of inflorescence provide sinks and cause increase in proteolysis in older leaves (Richards and Templeman, 1936; Mason and Maskell, 1929–1930) but under these conditions the rate of proteolysis is increased when such plants are transferred to darkness whereby carbohydrates reserves become low (Nightingale, Schermerhorn and Robbins, 1928). The possibility of an intrinsic factor, causing decline in rate of protein synthesis in older leaves, and not related to carbohydrate or N–sources cannot be overlooked (Mothes, 1926; Walkley, 1940; Walkley and Petrie, 1941). In much of the work described above detailed carbohydrate analyses are not given, but such data as exist, suggest that the plants, grown under glasshouse conditions, are relatively low in carbohydrates which consequently may act as limiting factors in protein formation.

In starving leaves in darkness many investigators have found that decrease in amount of protein is associated with decrease in amount of carbohydrate. Spoehr and McGee (1923) and Deleano (1912) showed increases in organic nitrogen when starving plants in darkness were transferred to glucose solutions.

From a review of such experimental data, together with some experimental work of his own, Paech (1934) put forward the view, which has gained some acceptance, that the protein content is determined by the total ammonia and monosaccharide contents in a simple mass law equilibrium.

There exists a body of evidence not compatible with the simple view of Paech. Disagreement exists on the so-called "protein-sparing" action of carbohydrates during starvation. The work of Smirnov (1923), Mothes (1926), Yemm (1937), Vickery *et al* (1933) shows that under starvation conditions the amount of protein decreases simultaneously with decrease in carbohydrates; whilst that of Deleano (1912) and Meyer (1918) showed that proteolysis did not occur until partial depletion of carbohydrate supplies occurred. Kabos (1936) found that protein increased in amount in *Sinapis* plants in the dark in solutions containing up to 2 p.c. sucrose but that further increase in the sugar concentration up to 8 p.c. caused no further increase in amount of protein-N.

Several workers (Thomas, 1932; Eckerson, 1924; Kraus and Kraybill, 1918; Nightingale and Robbins, 1928) have shown that when ammonium salts are supplied to nitrogen deficient plants (which are always high in carbohydrates) there is a marked increase in protein content and that the carbohydrate content becomes low. Amos and Wood (1939) showed that there is a high negative correlation between content of protein (and of total organic-N) and carbohydrates; and that hexoses, sucrose, fructosan and hemicellulose in grass leaves all contribute separately to protein formation. These results suggest that the carbohydrate status of the plant (i.e. whether carbohydrates are in excess or are limiting assimilation of ammonia) must be taken into account; and that no simple changes in accordance with the mass law take place.

It is clear that the N-status of the plant must also be considered in conjunction with possible relations between proteins and carbohydrates. Vickery, Pucher, Schoenheimer and Rittenberg (1939) have shown that by administering NH_4Cl containing N^{15} to plants there is a rapid replacement of part of N^{14} of the leaf protein with the isotope. In N-deficient, carbohydrate-high plants,

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[1] Papers I–IV of this series appeared in the Annals of Botany, 1938 and 1941.

[2] Dr. Petrie died after the completion of the experiment but before the data described in this paper were elaborated.

proteolysis occurs owing to presence of sinks but increase in amount of protein associated with decrease in amount of carbohydrate occurs on addition of ammonia (Mothes, 1926; Amos and Wood, 1939).

In this paper we consider how on theoretical grounds, inter-relation may arise between protein on the one hand and carbohydrates and ammonia on the other and record an experiment designed to submit the existence of the inter-relationship to a more critical test.

POSSIBLE LINES OF PROTEIN SYNTHESIS AND POSSIBLE MECHANISMS OF THE INTER-RELATION WITH CARBOHYDRATES.

In papers I and III of this series (Petrie and Wood, 1938a and b) a simple hypothesis of a relationship between proteins and the total amino acids of the cell was adopted for convenience in ordering the data. Our considerations were based on the assumption that proteins are synthesized from and hydrolyzed back to amino acids. In coming now to consider the manner in which carbohydrates may be inter-related with protein synthesis a wider range of possibilities has to be brought into the field. Accordingly the following alternative hypotheses, all of which are implicit in literature on the subject, are set up:

(a) Reversible formation from the general mass of amino acids in the cell.

(b) Formation from a special set of amino acids formed at or attracted to special seats of synthesis as suggested by Block (1938). The main mass of amino acids in the cell may then arise by synthesis and by hydrolysis from proteins, a process that can occur in the cytoplasm; but they do not determine the amount of protein in the cytoplasm.

(c) Formation in the cytoplasm as a whole, as in (a) or at special seats as in (b), not from amino-acids but along another path.

According to hypothesis (a), a steady state may be conceived between amino acids and protein as already discussed in papers I and III (*loc. cit.*). In the case of hypothesis (b), protein could be related in practice only to the ultimate reactants, *viz.* ammonia and carbohydrate. In the case of hypothesis (c) if amino acids arise only by hydrolysis of proteins a steady state may be conceived as in (a) above; but if amino acids are formed from another source as well as from proteins then protein may be related only to ammonia and carbohydrate as in (b). But in any hypothesis it is conceivable that amounts of certain carbohydrates may be a factor determining the first stage of the process.

Considering the inter-relations of proteins and amino acids with carbohydrates several possible mechanisms arise: if hypothesis (a) or (c) is correct the steady state between proteins and amino acids may be independent of carbohydrate content except insofar as carbohydrate determines the amount of amino acid or protein, or the steady state may differ according to the carbohydrate concentration. There are two possible ways in which the latter may be brought about. First, as suggested by Yemm (1937), carbohydrates and amino acids may compete as substrates in respiration for activated oxygen. Then it is possible that certain amino acids are more readily deaminated than others. In this case, if the carbohydrate content is low and consequently amino acid respiration is appreciable certain amino acids may disappear more rapidly than others and therefore the proportions among the amino acids would be changed. Conditions would then exist for the establishment of a new steady state at which for a given total amount of amino acids there would be a smaller amount of protein. In this case the steady state would differ according to the carbohydrate content and consequent rate of deamination.

The second possibility is that carbohydrates, through respiration rate, may affect the rate of energy supply for the maintenance of the steady state.

DESCRIPTION OF EXPERIMENT.

Material. Seeds of a pure line of *Phalaris tuberosa* L. were grown in jars containing 3·8 kg. water washed sand and maintained at 70 p.c. saturation capacity by addition of distilled water. Fourteen days after sowing the same nutrients were applied as set out in Paper I of this series,

TABLE 1.

Results of Experiment.

Day 0		*Day* 0		*Day* 0	
NH_3–N	6	Resid. amino–N	71	Glucose × 10^{-1}	131
2 × amide–N	25	Protein–N × 10^{-1}	272	Fructose × 10^{-1}	26
Glutamine–N	n.d.	Water	537	Resp. rate	n.d.
Asparagine–N	n.d.	Total carbohydrates × 10^{-1}	612		
Total amino–N	83	Sucrose × 10^{-1}	455		

	Treatment						
Day 3	S0N0	S1N0	S2N0	S1N1	S2N1	S0N1	S0N2
NH_3–N	7	8	8	13	21	12	96
2 × amide–N	20	30	48	62	62	109	117
Glutamine–N	15	17	18	21	27	32	23
Asparagine–N	5	13	30	41	35	77	94
Total amino–N	101	94	119	172	143	180	224
Resid. amino–N	91	79	95	141	112	126	166
Protein–N × 10^{-1}	223	211	220	228	229	265	253
Water	548	495	547	546	512	575	568
Total carbs. × 10^{-1}	1,579	1,867	1,275	905	1,506	1,174	1,041
Sucrose × 10^{-1}	1,259	1,532	1,035	636	1,132	897	760
Glucose × 10^{-1}	61	71	36	28	150	17	1
Fructose × 10^{-1}	259	264	205	241	324	260	282
Resp. rate	1·89	1·91	1·87	2·61	2·02	2·10	2·83

	Treatment						
Day 4	S0N0	S1N0	S2N0	S1N1	S2N1	S0N1	S0N2
NH_3–N	9	8	9	10	9	10	47
2 × amide–N	16	19	20	33	39	101	105
Glutamine–N	10	11	12	23	18	31	39
Asparagine–N	6	8	8	10	21	70	66
Total amino–N	99	109	122	186	148	230	279
Resid. amino–N	91	99	112	169	128	180	226
Protein–N × 10^{-1}	196	183	183	222	197	262	252
Water	533	467	482	505	473	538	505
Total carbs. × 10^{-1}	1,472	1,595	1,806	1,504	1,715	1,227	1,029
Sucrose × 10^{-1}	1,153	1,133	1,254	1,079	1,104	896	769
Glucose × 10^{-1}	55	171	215	121	207	57	1
Fructose × 10^{-1}	265	291	338	304	404	274	260
Resp. rate	2·10	1·99	1·92	2·36	2·13	2·57	2·75

	Treatment						
Day 5	S0N0	S1N0	S2N0	S1N1	S2N1	S0N1	S0N2
NH_3–N	8	7	7	8	39	11	19
2 × amide–N	17	18	30	75	77	141	188
Glutamine–N	12	10	5	16	23	30	39
Asparagine–N	5	8	25	59	54	111	149
Total amino–N	58	61	84	124	154	245	302
Resid. amino–N	50	52	69	86	115	175	208
Protein–N × 10^{-1}	201	162	160	197	165	273	272
Water	534	424	442	464	429	539	560
Total carbs. × 10^{-1}	1,565	1,525	1,739	1,703	1,757	1,361	1,109
Sucrose × 10^{-1}	1,252	1,121	1,164	1,254	982	1,035	835
Glucose × 10^{-1}	74	165	251	149	285	74	40
Fructose × 10^{-1}	240	239	325	300	489	251	234
Resp. rate	1·85	1·90	1·83	2·28	2·30	2·34	2·40

Nitrogen fractions and carbohydrates in mg. per 100 gm. dry weight.
Water in gm. per 100 gm. dry weight.
Respiration rate in mg. CO_2 per gm. per hour.

with the addition of 0·0003 gm. $MnSO_4$ per pot. Three weeks after planting the seedlings were thinned to four even plants per pot. Seeds were planted on 4 July 1938, and differential treatments were applied on 7 October 1938.

Differential treatment. When the plants were ready for the experiment the pots were placed in cabinets under the constant conditions described previously (Petrie and Wood, 1938a). Analyses were performed on plants from four pots selected at random at the time of entry of other pots into the cabinets; these are designated Day 0. Treatments were applied to the other pots at the time of entry into the cabinets and batches were removed for analyses 3, 4 and 5 days after entry into the cabinets.

There were seven treatments including the control designated: S0N0, S0N1, S0N2, S1N0, S1N1, S2N0, S2N1. S0 plants received no sucrose; S1 plants received 2 gm. sucrose per pot; and S2 plants 5 gm. sucrose per pot: N0 plants received no additional nitrogen; N1 plants received 1 gm. NH_4Cl per pot; and N2 plants 2 gm. NH_4Cl per pot. Four pots from each of the seven treatments were harvested on each of days 3, 4 and 5. Three plants from each pot in any one treatment were used for analysis, the remaining plants were used for measurement of respiration rate.

Analytical methods. The leaves were harvested as previously described (Petrie and Wood, 1938a). Samples for determination of N–compounds were prepared and protein, ammonia, total amides and total amino–N estimated as previously described. Glutamine was estimated by by hydrolyzing an aliquot at pH 6·5 followed by distillation *in vacuo* with borax-NaOH buffer. Estimations of sucrose, glucose and fructose were carried out in fresh material using the procedures described by Amos and Wood (1939). Measurements of respiration rate were made by the conductivity method described by Wood (1941); this method enabled the rate to be determined at frequent intervals and greatly simplified extrapolation to the time of cutting of analytical samples.

Analyses and measurement of respiration rates in all cases were performed on duplicate samples of the original material. Using the mean value results are expressed on a dry weight basis. The results are given in Table 1 and Figs. 1 and 2.

DISCUSSION OF RESULTS.

Dry weight data. These are presented as the mean value per pot, together with analysis of variance in Table 2.

TABLE 2.

Mean dry weights per pot (gm.).

Day 0 3·35

	Treatment							
	S0N0	S1N0	S2N0	S0N1	S1N1	S2N1	S0N2	Day mean
3	3·87	4·01	3·42	3·61	3·93	3·70	3·57	3·73
4	4·04	4·29	3·89	3·83	3·89	3·82	3·74	3·93
5	4·12	4·43	3·77	4·40	4·05	3·91	3·94	4·09
Treatment mean	4·01	4·24	3·69	3·94	3·95	3·81	3·75	3·91

Analysis of Variance on data for days 3–5.

Interaction insignificant; time and treatment significant at 1 p.c. point.
Coefficient of variation = 4·67 p.c.
Standard error for comparing treatment means = ± 0·1056.
Standard error for comparing day means = ± 0·0691.
Treatment mean of S1N0 is the only value that can be shown to be significantly different from the general mean.

Effects of sucrose treatments on amount of carbohydrates in leaves. Examination of Table 1 and Figs. 1 and 2 shows: (1) transference of the pots from the conditions of the glasshouse to the constant illumination of the cabinet caused a marked increase in amount of carbohydrates. The increase is most marked in the case of sucrose and fructose, but in general the amount of glucose is also increased, (2) total carbohydrates increased in each separate N-treatment with increased application of sucrose to the soil, except in treatment S2N0 and S1N1 on day 1 when a steady state was possibly not attained, (3) Fig. 1 shows that there is a general tendency for sucrose, glucose and fructose to follow the same trends. With increased application of sucrose in each nitrogen treatment the amount of sucrose shows fluctuations but glucose and fructose increase in amount in each case. (4) Increased external application of sucrose in each case is associated with a marked reduction in water content of the leaves, probably brought about by an osmotic effect at the roots. This technique has allowed variation in water content to take place other than by the application of large amounts of ammonium salts as described in papers I and III of this series.

Relations of protein with carbohydrates and ammonia. It is apparent from examination of Figs. 1 and 2 that differential nitrogen treatment profoundly alters the amounts of carbohydrates. If total organic–N (protein–N *plus* amino acid–N *plus* amide–N) is plotted against total carbohydrate (sucrose *plus* hexoses), on each day at each separate sucrose treatment increased nitrogen treatment results in a decrease in total carbohydrate. The scatter of the data is such that they may be expressed as a single correlation coefficient ($r = -\cdot760$; P[3] $< \cdot01$). If amount of protein is plotted against total or individual carbohydrates it is clear that increased N-treatment causes an increase in amount of protein and decrease in amount of carbohydrates. The correlation coefficient of protein on total carbohydrates is $r = -\cdot759$; $P < \cdot01$ on sucrose is $r = -\cdot568$; $P < \cdot01$; on glucose is $r = -\cdot776$; $P < \cdot01$; and on fructose is $r = -\cdot466$; $P = \cdot02$. These results are in agreement with the data of Amos and Wood (1939).

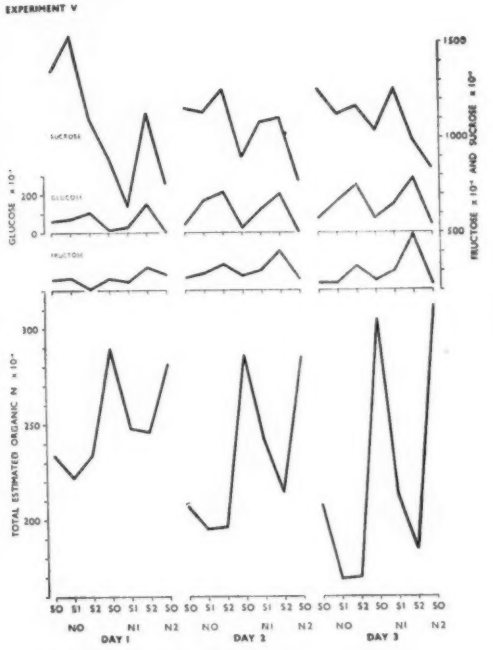

Fig. 1. Treatment effects on the contents of carbohydrates and total organic nitrogen in the leaves. Values are given in mg. per 100 gm. dry matter.

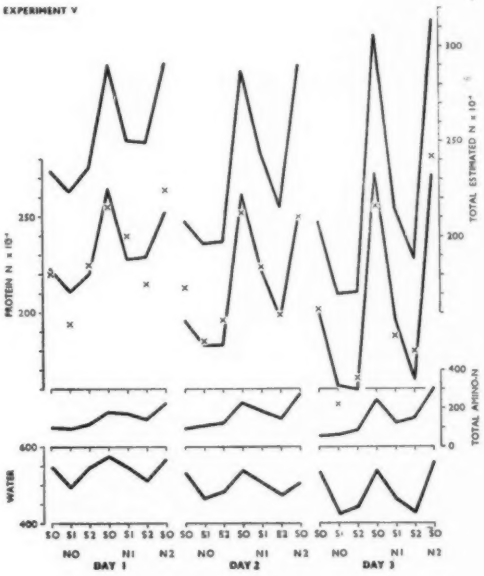

Fig. 2. Treatment effects on the content of nitrogen compounds and water in the leaves. The nitrogen values are given in mg. per 100 gm. dry matter and the water values in gm. per 100 gm. dry matter. Crosses indicate values for protein calculated from equation (5), Table 3.

In Fig. 3 protein content is plotted against sucrose and for each point the values for water and ammonia content are given. It is apparent from Table 1 that amount of protein increases with increased application of ammonium salt to the soil and the aforementioned experiments of Vickery *et al.* (*loc. cit.*) with N^{15} salts show that this isotope of nitrogen quickly enters the protein molecule. But neither in this experiment nor in those described previously by us does amount of protein show a significant correlation with the ammonia content within the plant. In the experiment described here the correlation of protein on ammonia–N ($r = +\cdot266$) is significant only at the 15 p.c. point and that on the logarithm of ammonia–N ($r = +\cdot366$) is significant only at the 10 p.c. point.

We have calculated the regression of protein on sucrose, water and ammonia contents. The equation takes the form $P = a - b_1, Su + b_2U + b_3N$ (equation 1, Table 3). The equation

[3] P is the probability that a given correlation should arise, by random sampling, from an uncorrelated population.

accounts for 84·1 p.c. of the total variance (V) of P but the coefficient of the ammonia term is insignificant and its removal scarcely alters the value of V (equation 2, Table 3). Introduction of a term for the logarithm of ammonia is also insignificant and considerably decreases the value of V (equation 3, Table 3).

It would appear therefore that under the conditions of our cabinet experiments, one of the features of which is the high carbohydrate content of the plants, there is no simple relation of protein to carbohydrates as suggested by Paech (1935). It is probable, as suggested by the previous survey of the literature, that only when amount of carbohydrate is low is there a linear relation in the same direction between proteins and carbohydrate. Furthermore, there is no evidence in our experiments of a relationship between protein and ammonia–N content of the cells.

Relation between protein and amino-acids. Residual amino acid content (total amino–N *minus* amino–N of amides) is correlated with both sucrose ($r = -\cdot723$; $P < \cdot01$) and with ammonia–N ($r = +\cdot591$; $P < \cdot01$). The regression of amino-acid on sucrose and ammonia is given in equation 4, Table 3. Inclusion of a term for water content gives an insignificant coefficient and does not alter the value of V. It is obvious that the regression equation of protein on sucrose, water and ammonia given in the last section (equation 1, Table 3) may take the form $P = a + b_1A + b_2U$. The regression equation for protein on total amino–N and water (equation 5, Table 3) accounts for 90·3 p.c. of the variance of protein and the equation for protein on residual amino–N and water (equation 6, Table 3) for 87·3 p.c. of the variance. Equations of this type in earlier experiments described by us (Petrie and Wood, 1938) have always accounted for a large percentage of the total variance of protein.

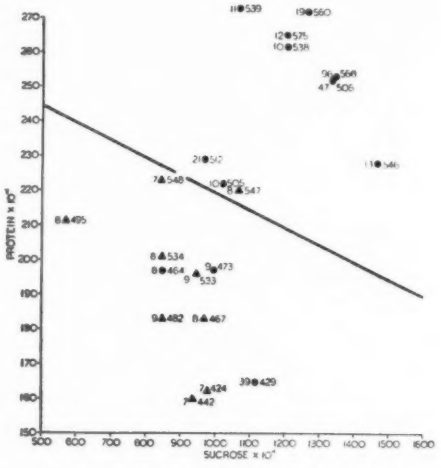

Fig. 3. Protein content plotted against sucrose content. The corresponding values of water (to right of each point) and of ammonia (to left of each point) are given for each point. The line is that of equation (1) Table 3 for the mean values of water and ammonia.

Alternative hypotheses of protein synthesis. The relations between proteins and amino acids established in the last section appear to exclude hypothesis (b) in the second section of this paper. We are faced therefore with the alternative hypotheses of formation along an unknown path from ultimately a carbohydrate and ammonia or formation from preformed amino acids. It is clear that no simple mass-law relation exists between proteins on the one hand and a carbohydrate and ammonia on the other as suggested by Paech (1935). It is unlikely that hexoses provide a direct substrate for protein synthesis but rather that intermediary products of glycolysis or acids formed by the oxidation of these during respiration are utilized. Barker (1936) has shown in potatoes that the curve relating respiration rate and carbohydrates approaches an asymptote when a certain amount of sugar is present in the cells. The fact that protein increases in amount whilst carbohydrates decrease may not therefore preclude a direct synthesis of protein, for it is possible that rate of formation of respiratory substrates remained approximately constant with falling total carbohydrate content.

The relations between proteins and ammonia present difficulties for the direct synthesis hypothesis. If the carbohydrate substrate remained constant, mass-law considerations would expect increased ammonia content to be associated with increased protein content but such association is not found in our experiments. To account for a direct synthesis ancillary assumptions must be introduced.

Since formation of proteins necessitates an increase in free energy, one possibility is that a given amount of respiratory energy is capable of maintaining a definite amount of protein. Under such circumstances addition of ammonia might lead to increased protein content without detectable increases in ammonia content; when ammonia was in excess, amount of protein would be determined by the amount of respiratory energy. Our data do not support the latter conclusion.

TABLE 3.

Regression Equations.

All the regressions given are significant at or below the 5 p.c. point.

Coefficients significant at or below the 5 p.c. point are given in bold type, those insignificant in italic type. P = protein nitrogen content $\times 10^{-1}$; A_T = total amino-N content; A_R = residual amino-nitrogen content; U = water content; N = ammonia nitrogen content; Su = sucrose content $\times 10^{-1}$; R = respiration rate; V = percentage of the variance of P or A ascribable to the average effect of the independent variables. The whole of the data for days 3, 4 and 5 were used in calculating the regressions.

		V
(1)	$P = -77{\cdot}5 - (\mathbf{{\cdot}050 \pm {\cdot}021})\ Su + (\mathbf{{\cdot}682 \pm {\cdot}078})\ U - (\mathit{{\cdot}011 \pm {\cdot}058})\ N$	84·1
(2)	$P = -77{\cdot}7 - (\mathbf{{\cdot}050 \pm {\cdot}021})\ Su + (\mathbf{{\cdot}682 \pm {\cdot}084})\ U$	84·9
(3)	$P = -48{\cdot}8 - (\mathbf{{\cdot}037 \pm {\cdot}035})\ Su + (\mathbf{{\cdot}576 \pm {\cdot}106})\ U + (\mathit{10{\cdot}78 \pm 17{\cdot}75})\ \log N$	66·2
(4)	$A_R = 238{\cdot}9 - (\mathbf{{\cdot}152 \pm {\cdot}009})\ Su + (\mathbf{39{\cdot}56 \pm 5{\cdot}94})\ \log N$	52·1
(5)	$P = -62{\cdot}2 + (\mathbf{{\cdot}277 \pm {\cdot}041})\ A_T + (\mathbf{{\cdot}465 \pm {\cdot}061})\ U$	90·4
(6)	$P = -77{\cdot}9 + (\mathbf{{\cdot}359 \pm {\cdot}064})\ A_R + (\mathbf{{\cdot}493 \pm {\cdot}069})\ U$	87·3

Interpretation of the significance of the water term in equation 1, Table 3, is obscure. On the direct synthesis hypothesis, the decrease in protein content associated with decrease in water content might be explained by increased utilization of one of the reactants, presumably the sugar, along another path as its concentration increases; but there is no experimental evidence to suggest such a phenomenon.

It is apparent that the hypothesis of synthesis of proteins by a path other than from amino acids requires ancillary assumptions of a speculatory nature, some of which are not supported by the experimental evidence; and it must be concluded that the weight of evidence is against synthesis along such a path.

On the other hand synthesis of proteins from the bulk of the amino acids is supported by the evidence of all our experiments. This consists in a definite relation between amino acid content, ammonia and carbohydrate contents, and a definite relation between protein, amino acid and water contents which may be interpreted along the lines suggested in papers I and III of this series (Petrie and Wood, 1938a and b).

The effect of carbohydrate content on the relations between proteins and amino acids. The form of the relation between concentrations of protein and amino acids is such that the curve is concave to the amino acid axis. One explanation of this effect advanced in paper III (*loc. cit.*) of this series was that some amino acids are oxidized more rapidly than others. We explore here only the possibilities outlined in the second section of the present paper, viz. that carbohydrate content may determine the form of the protein-amino acid relation by (i) amino-acids competing with carbohydrates as respiratory substrates and (ii) carbohydrates, through respiration rate, determining the rate of energy supply for maintenance of the steady state.

We have obtained the differences between the observed value for protein and that calculated from regression equation 6, Table 3, and calculated the correlation coefficient of these differences on individual carbohydrates. In no case is the correlation significant.

The data show no more than the fact that carbohydrates are utilized in synthesis of organic nitrogen compounds and under the conditions described here decrease in amount as protein and amino acids increase in amount.

Concerning suggestion (ii) above, that carbohydrate content through respiration rate may determine the steady state relations between proteins and amino-acids, it will be shown in a subsequent paper that respiration rate is probably a parameter determining these relations. But for the present we restrict ourselves to showing that respiration rate is not related to carbohydrate content, for the correlation coefficient between respiration rate and individual carbohydrates is negative (e.g. for sucrose $r = -\cdot 811$; $P < \cdot 01$).

There is therefore no evidence in the experiment described here that the steady state relations between proteins and amino acids may differ according to the carbohydrate concentration.

SUMMARY.

An experiment is described wherein plants of *Phalaris tuberosa* L. were subjected to differential sucrose and nitrogen treatments in a constant environment. Within the same nitrogen treatment increased external applications of sucrose resulted in an increase in amount of carbohydrates within the plant. Within the same sucrose treatment increased applications of ammonium salts resulted in a decrease in amount of carbohydrates. High external applications of sucrose were associated with decrease in water content of the plants.

Under the conditions described here it is shown that protein content shows no relationship with carbohydrates as expected from a simple mass-law relationship for all carbohydrates decrease in amount as protein content increases. There is also no relation between amount of protein and amount of ammonia present within the cell, although ammonia is limiting and the amount of protein increases up to a point with increased external supply of ammonia. On the other hand 90 p.c. of the variance of protein is accounted for in terms of amino-acid and water contents. It is suggested that the balance of evidence indicates that proteins are synthesized from the whole of the amino acids within the cell rather than along any alternative path.

The inter-relations between protein, amino acids and water contents are not affected by changes in amounts of the carbohydrates, sucrose, glucose and fructose.

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STUDIES ON THE NITROGEN METABOLISM OF PLANTS

VI. INTER-RELATIONS AMONG RESPIRATION RATE, CARBOHYDRATES, SOLUBLE NITROGEN COMPOUNDS AND PROTEIN IN LEAVES

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(Accepted for publication 30th July, 1942.)

INTRODUCTION.

In paper II (Wood and Petrie, 1938) of this series certain relations were shown to exist in the leaves of grasses between soluble nitrogen compounds and respiration rate. In that paper it was stated that interpretation of these relations required data for carbohydrate contents which were not available for the experiments therein described. Furthermore, it was felt that better figures for respiration rate should be obtained than those given in the previous experiments. In the latter, respiration rate was measured over three consecutive one-and-a-half hour intervals by absorbing carbon dioxide in Reisert towers and subsequent extrapolation of the curves so obtained to time of cutting of the samples.

In this paper we utilize the data of an experiment presented in Table 1 of paper V of this series (Wood and Petrie, 1942). In this experiment amounts of sucrose, glucose and fructose were determined for each treatment; glutamine in addition to total amides was estimated; and frequent readings of respiration rate were made possible by using the conductivity method described by Wood (1941).

THE AMINO ACID CONTENT.

In paper V of this series evidence was advanced which suggests that proteins are synthesized from amino acids rather than along an alternative path. We may expect, therefore, that the concentration of amino acids should be related to that of their precursors. Recent work (Adler, 1938; Euler, 1938) suggests that the precursors are keto-acids, such as the respiratory "mediators", and ammonia. The carbohydrates, but especially sucrose which is especially utilized in glycolysis (James, 1940), might be a measure of the carbon-containing precursor.

The correlation coefficient of residual amino–N on sucrose is $r = -\cdot 723$; $P^1 < \cdot 01$ and on glucose is $r = -\cdot 421$; $P < \cdot 05 > \cdot 02$. However, the correlation between amino–N and fructose ($r = -\cdot 050$) is quite insignificant; this result is in agreement with previous work of Amos and Wood (1939) where significant relations between fructose and nitrogen compounds could not be demonstrated. It is apparent that furanose forms are more readily utilized than pyranose forms in nitrogen metabolism and the data show that fructopyranose may accumulate whilst sucrose (a fructo-furanose) is disappearing (cf. James and James, 1940).

In each case the correlation coefficient of residual amino acids on sucrose and glucose is negative indicating that these carbohydrates are consumed in amino acid formation but that no mass-law relationship exists between them. However, sucrose concentration is not necessarily a measure of carbohydrate substrates in respiration, for Barker (1935) has shown in potatoes that the relation between respiration rate and sucrose is of hyperbolic form as expected from the Michaelis equation, so that at high sucrose concentrations respiration rate is practically independent of the sucrose concentration. It has been pointed out in paper V of this series that a characteristic of the experiment here described was the very high sucrose content of plants subjected to the cabinet treatment. We shall present evidence in a section below that respiration rate remains constant in plants not treated with additional nitrogen, although the carbohydrate content varied considerably. It is probable therefore that the concentration of the glycolytic product, or its oxidation product, which might be a precursor of amino acids, remained constant throughout the experiment.

The curve relating residual amino acids to ammonia consists of a part wherein amino–N increases steeply in a linear manner with increase in ammonia–N and a region wherein amino–N

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[1] P is the probability that a given correlation should arise, by random sampling, from an uncorrelated population.

content shows little increase with large increase in ammonia content. Although not necessarily a logarithmic function, for convenience in ordering the data the relationship may be expressed by plotting amino–acid–N against the logarithm of the ammonia content.

The correlation coefficient of amino acid on log. ammonia is $r = + \cdot 591$; $P < \cdot 01$. If the three high values for ammonia which form the asymptotic limb of the curve are excluded there is a high linear correlation between amino acids and ammonia ($r = + \cdot 781$; $P < \cdot 01$). The regression of amino acid on sucrose and log. ammonia (equation (1), Table 1) is significant and yields significant coefficients, but sucrose and ammonia contents are correlated and the equation may be expressed simply in the form $A_R = a + b \log N$. Addition of other variables, including water content gives insignificant coefficients (equation (2), Table 1).

TABLE 1.

Regression equations.

The regressions given are significant at or below the 5 p.c. point.

Coefficients significant at or below the 5 p.c. point are given in bold type, those insignificant in italic type.

A_R = residual amino–N content; Su = sucrose content; U = water content; N = ammonia–N content; OR = respiration rate; V = percentage of variance ascribable to the average effect of the independent variables.

	V
(1) $A_R = a - (\mathbf{\cdot 152} \pm \cdot 0096)\,Su + (\mathbf{39 \cdot 56} \pm 5 \cdot 94) \log N$	52·1
(2) $A_R = a - (\mathbf{\cdot 130} \pm \cdot 063)\,Su + (\mathit{\cdot 175} \pm \cdot 190)\,U + (\mathit{36 \cdot 28} \pm 31 \cdot 70) \log N$	49·3
(3) $OR = a + (\mathbf{\cdot 00526} \pm \cdot 000059)\,A_R - (\mathit{\cdot 00172} \pm \cdot 00097)\,U$	66·3
(4) $OR = a + b\,A_R$	

Similar relationships between amino acids and ammonia of this simple form have been obtained in all our experiments (Wood and Petrie, 1938) and indicate that under the conditions of our experiments ammonia content initially behaves as a limiting factor in amino acid formation.

Interpretation of the limiting effect may be made by reference to a specific example. Euler *et al* (1938) and Adler *et al.* (1938) have shown that in the presence of glutaminic dehydrase glutamic acid is hydrolyzed almost completely into ammonia and α–ketoglutaric acid (which is one of the "respiratory mediators"), but in the presence of Co–enzyme I and provided carbohydrate glycolysis is proceeding simultaneously, glutamic acid is formed from the other two products and a position of equilibrium is attained. Commencing with glutamic acid alone the position of equilibrium will be determined by the relation: $\dfrac{[\alpha\text{–ketoglutaric acid}]_e \times [\text{ammonia}]_e}{[\text{glutamic acid}]_e} = K$

where the terms in square brackets denote equilibrium concentration terms. But under the conditions of the experiment described here the initial ammonia concentration is low and α–ketoglutaric acid concentration is maintained constant by continuous breakdown of carbohydrates in glycolysis. We have therefore an "asymmetrically decomposed system" (Lewis, 1920) in which the products are not present in equivalent quantities. With the addition of ammonia to this system both ammonia and glutamic acid contents will increase (although the increase in ammonia content may be small) until true equilibrium concentrations are attained where the products are present in equivalent quantities. At this point no further increase in glutamic acid will occur with further increase in ammonia and the α–ketoglutaric acid may become limiting.

GLUTAMINE AND ASPARAGINE CONTENTS.

Both glutamine and asparagine are highly correlated with residual amino–acid–N. The correlation coefficient of glutamine on residual amino–N is $r = + \cdot 862$; $P < \cdot 01$ and of asparagine on residual amino–N is $r = + \cdot 710$; $P < \cdot 01$. Both amides show significant negative correlations with carbohydrates and positive correlations with the logarithm of the ammonia content. It follows also that glutamine and asparagine exhibit similar relationships with protein as does residual amino acid–N. This was reflected in regression equations (5) and (6) in Table 2 of paper V of this series.

It would appear to be a reasonable hypothesis that glutamine exhibits equilibrium relations with glutamic acid and asparagine with aspartic acid, both of which form a fraction of the total amino acids.

RESPIRATION RATE IN RELATION TO OTHER VARIABLES.

In Fig. 1 respiration rate is plotted against sucrose content and for each point values for residual amino acid–N and for water contents are given. It is apparent that respiration rate

increases with decrease in sucrose content, with decrease in water content and with increase in amino acid content.

The correlation coefficients of respiration rate on individual carbohydrates are of the same order of magnitude and significance as those of residual amino–N on the same carbohydrates. The coefficient for respiration rate on sucrose is $r = -\cdot 811$; $P < \cdot 01$, on glucose $r = -\cdot 423$; $P = \cdot 05$ and on fructose there is no correlation ($r = +\cdot 002$). As in the case of amino acid formation, it is apparent that furanoses are consumed preferentially in respiration.

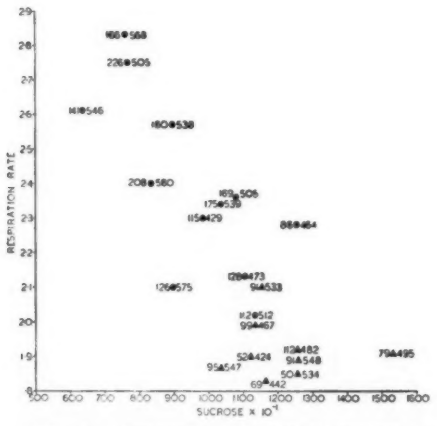

Fig. 1. Respiration rate plotted against sucrose content. For each point corresponding values of residual amino–N (to left of point) and of water contents (to right of point) are given. Units as in Table 1 of paper V of this series.

The negative correlation between respiration rate and sucrose is explicable in terms of Barker's (1935) findings mentioned previously, viz., that at high sugar concentrations respiration rate remains approximately constant, the substrate produced by glycolysis being limited by the enzyme system. Indications that respiration rate is independent of sucrose content can be seen in Fig. 1. In this figure points for the N0 series (i.e. treatments in which no additional nitrogen was supplied) are marked by triangles. The value of amino acid does not vary greatly for these points and it may be seen that respiration rate for this series is approximately constant over a relatively wide range of sucrose content.

The regression of respiration rate on residual amino–N and water content is given in equation (3) Table 1. The coefficient of the amino acid term is positive and that for the water term negative. Although the coefficient for the water term is insignificant its removal from the regression decreases the value of V (see Table 1). Amino acid and water contents are correlated and the water content must take up some of the variance of amino acid. Similar regression for respiration rate on amino acid and water contents have been obtained in experiments I and II of this series (Wood and Petrie, 1938).

It is apparent therefore that respiration rate increases as amino acid concentration increases and that in this experiment this is superimposed on a constant rate of respiration due to oxidation of glycolytic products.

There remain for consideration effects of respiration rate on relations between proteins and amino acids.

Respiration rate is positively correlated with protein content ($r = +\cdot 585$; $P < \cdot 01$) but examination of the data shows that the relationship is more complex than that suggested by the coefficient. It is apparent that the effects of amino acid and water contents as determinants of respiration rate and the separate effects of these two variables as determinants of protein must be brought into the picture. It has been shown in paper V of this series (Wood and Petrie, 1942) that the regression of protein on amino acid and water contents accounts for 90 p.c. of the total variance of protein. We have calculated the values of protein from equation (6) of Table 3 of that paper and also the correlation coefficient of the difference between observed and calculated values on respiration rate. The correlation is insignificant as might be expected since respiration rate is highly correlated with amino acid content.

The relations between the variables here considered are such as are not easily expressed by regression functions and are shown graphically in Figs. 2 and 3. In Fig. 2 protein is plotted against residual amino acid and for each point values for water content and respiration rate are given; the line is that of equation (6) in Table 3 of Paper V of this series. In Fig. 3 respiration rate is plotted against residual amino acid content and for each point values for protein and water contents are given; the line is that of equation (3) in Table 1 of this paper.

In Fig. 2 the scatter of the data is different from that described for experiments in previous papers of this series in that differential sucrose treatment results in a decrease in water content over the whole range of the data. In previous experiments low water content always resulted from high ammonium salt treatment and consequently was always associated with high amino acid content. In Fig. 2 the regression line divides the data into two regions; that below the line having water contents from about 430–500 and that above the line from 500–570. At each of these two approximately constant water contents it is clear that respiration rate increases with amino

acid content and also with protein content, although it must be borne in mind that protein content is positively correlated with amino acid content.

In Fig. 3 where respiration rate is plotted against amino acid content the relation with protein is more clearly seen and it is evident that at any particular amino acid content respiration rate tends to increase with decreased water content and with decreased protein content.

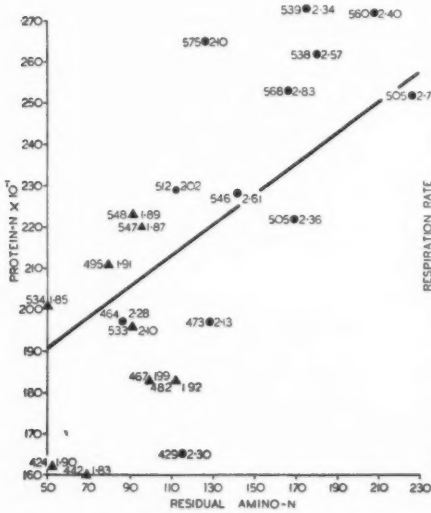

Fig. 2. Protein content plotted against residual amino–N. For each point corresponding values of water content (to left of point) and of respiration rate (to right of point) are given. The line is that of equation (6) in Table 3 of paper V of this series for the mean value of water content. Units as in Table 1 of paper V of this series.

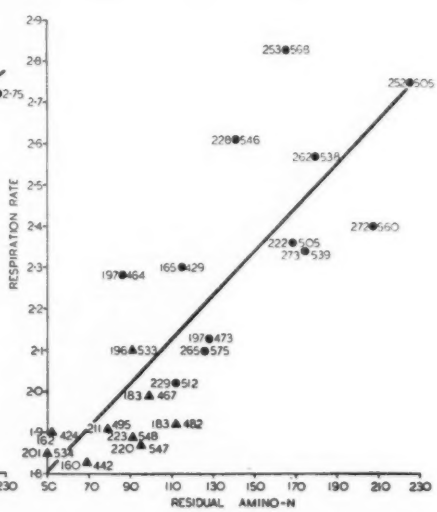

Fig. 3. Respiration rate plotted against residual amino–N. For each point corresponding values of protein (to left of point) and of water content (to right of point) are given. The line is that of equation (3) Table 1 of this paper for the mean value of water content. Units as in Table 1 of paper V of this series.

In explanation of these results two possibilities must be considered. First, amount of protein might be a measure of the number of "respiratory centres" within the cells or a measure of a component of the enzyme system. In this case respiration rate would be determined by protein content as well as by concentration of amino acids and carbohydrates. Richards (1938), Petrie and Williams (1938) and Wood (1941) record a high correlation between respiration rate and protein content determined at intervals throughout the life-cycle of plants, fluctuations being accounted for in terms of amino acid or carbohydrate contents. In Fig. 4 respiration rate on a protein basis (Rp) is plotted against amino acid concentration (obtained by dividing the amino acid content by the water content, both on a dry weight basis); there is no discoverable relation between these two variables, nor does Rp exhibit any discoverable relation with carbohydrate concentration. There is, therefore, no evidence, under the conditions of these experiments, that respiration rate is determined by the total amount of protein acting as respiratory centres. This does not mean that a portion of the protein may not behave in this way since Wood (1941) has advanced evidence that respiration rate shows a higher correlation with cytoplasmic protein than with total protein.

The second possibility in explanation of the inter-relations is simply that respiration rate increases with amino acid concentration (whether by increase in amount of amino acid or by decrease in water content) and that one or more amino acids are deaminated more readily than others. The consequences of changing proportions among different amino acids have been discussed more fully by Petrie and Wood (1938). Under these circumstances, since the amount of protein at equilibrium is determined by the products of the concentrations of the individual amino acids, the concentrations of amino acids more resistant to oxidation must increase, such increase taking place by hydrolysis of proteins; so that a higher total amino content is in

equilibrium with a lower protein content. It has been shown (Petrie and Wood, 1938) that at least one amino acid, viz. cystine does not increase when other amino acids increase due to reduction in water content. The data for the experiment described here are fully in accord with this hypothesis which we suggest as the most likely cause of the observed relations between proteins, amino acids and water, although confirmation must await determinations of amounts of individual amino acids under conditions similar to those of our experiments.

CONCLUSIONS AND SUMMARY.

It is possible to present a picture of the probable course of nitrogen metabolism in leaves from the experiments described in this series of papers wherein plants were supplied with amounts of nitrogen compounds and carbohydrates under constant conditions of light intensity, temperature, humidity and soil water content.

Under the conditions of the experiments amount of protein and of amino acids and amides are not correlated with changes in the same direction of carbohydrate content. On the contrary, increase in amount of protein and of soluble organic nitrogen compounds is associated with decrease in amounts of individual carbohydrates, all of which, but especially sucrose and glucose, may contribute separately to the formation of organic nitrogen compounds. It is concluded that an intermediary compound of carbohydrate degradation, either a glycolytic product or one of the oxidation products of these, which forms the starting point for synthesis or organic nitrogen compounds, is formed at a rate independent of carbohydrate concentration. It is realized that under the conditions of our experiments carbohydrate content was exceptionally high and it is probable that under other circumstances carbohydrates may "limit" organic nitrogen synthesis in which case amounts of protein and of amino acids and amides might exhibit a positive correlation with carbohydrate content.

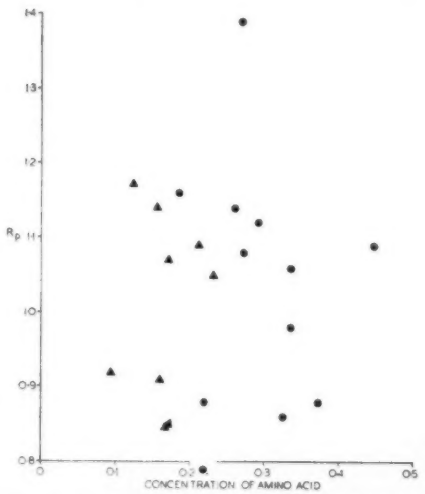

Fig. 4. Respiration rate on a protein basis (R_p) plotted against residual amino–N concentration. R_p obtained by dividing respiration rate (mg. CO_2 per gm. dry weight per hour) by amount of protein per 100 gm. dry weight. Amino–N concentration obtained by dividing residual amino–N (mg. per 100 gm. dry weight) by water content (gm. per 100 gm. dry weight).

In all experiments increased external application of ammonium salts resulted in increase of ammonia–N within the leaves. The amount of ammonia exhibits definite steady state relations with amino acids and also with the amides asparagine and glutamine. These compounds increase in amount with increase in ammonia content until a point is reached when further increase in ammonia is not associated with further increase in amount of amino acids or amides; at this point it is probable that the amount of carbohydrate intermediary is limiting.

External application of asparagine to plants does not bring about accumulation of the amide but results in increases in protein, amino acids and amides in conformity with relationships found to exist among these by increasing ammonia by means of external applications of ammonium salts.

Protein content exhibits no discoverable direct relation with ammonia content, but shows definite steady state relationships with both amino acid and water contents, and the balance of evidence suggests that proteins are formed from the whole of the amino acids within the cell rather than along an alternative path.

Increase in protein content is associated with increase in amino acid content and increase in water content. Respiration rate in all experiments is not positively correlated with carbohydrates but shows a high positive correlation with amino acid concentration. The evidence suggests that some amino acids are oxidized more readily than others bringing about a change in the proportions among the individual amino acids. Such differential oxidation is at least one factor accounting for the observed relations among protein, amino acids and water.

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STUDIES ON NATURAL RESISTANCE

1. THE RELATIVE RESISTANCE OF GUINEA-PIGS AND MICE TO DIPHTHERIA TOXIN.

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(Accepted for publication 8th October, 1942.)

It has been established that white mice and rats are highly resistant to diphtheria intoxication and infection (Roux and Yersin, 1888; Cobbett, 1899; Behring and Kitashima, 1901; Kolle and Schlossberger, 1920; Coca, Russell and Baughmann, 1921; Glenny and Allen, 1922; Jungeblut, 1927; Ramon, Noureddine and Erber, 1928; Ulrich, 1939).

Further, it has been shown that these animals are much more susceptible to the intracerebral injection of diphtheria toxin than to subcutaneous or intraperitoneal injection (Roux and Borrell, 1898; Bieling and Oelrichs, 1937). Following intracerebral injection damage occurs mainly in the nervous system proper; the characteristic signs which follow subcutaneous or intraperitoneal injection being either absent or much less marked.

Similar experiments with living diphtheria bacilli indicate that white mice are highly susceptible to intracerebral injection (Seligmann and Jungeblut, 1939; Frobisher and Parsons, 1940).

Various theories have been advanced to explain the natural resistance of white mice and rats to diphtheria toxin. It was considered by some workers that this phenomenon might be due to the presence of normal antitoxic antibodies in the blood of the mouse. It was found, however, that the serum of normal mice or rats could not protect guinea-pigs against diphtheria intoxication and that the blood of mice injected with diphtheria toxin contained enough free toxin to kill a guinea-pig (Aronsen, 1893; Koudrevetsky, 1893; Kuprianow, 1894; Cobbett, 1899; Goodman, 1907; Pettit, 1914; Kolle and Schlossberger, 1920; Coca, Russell and Baughmann, 1921; Sbarsky, 1926).

Pettit suggested that diphtheria toxin does not react with the tissues of the resistant animal, whereas a high affinity exists between the toxin and the tissues of susceptible species. He demonstrated that histologically the tissues of the resistant species are not altered by diphtheria toxin and that, in these animals, a large proportion of the injected toxin is excreted in the urine.

This view was supported by Ramon's observation that rats and mice could not be immunized by injection of anatoxin or unaltered toxin. This was later disproved by several workers who found that, although the immunity response of mice and rats after injection of diphtheria toxin or anatoxin is weak in comparison with the response of a susceptible animal, nevertheless a definite response can be obtained (Coca, Russell and Baughmann, 1921; Aronsen, 1893; Kuprianow, 1894; Kolle and Schlossberger, 1920; Ulrich, 1939).

In guinea-pigs suffering from diphtheria intoxication characteristic haemorrhagic changes occur in the adrenal and pituitary glands; such lesions are not found in rats or mice. In view of this, Ulrich considers that, owing to the unimpaired "functional efficiency" of the adrenal and pituitary glands in rats and mice, these animals are able to combat the deleterious effect of the toxin on the blood vessels.

This theory does not take into consideration the fact that damage to the adrenals and to the pituitary is by no means pathognomonic of diphtheria toxin.

So far no theory exists to explain adequately the phenomenon of natural resistance. With the object of throwing some light on this problem, studies have been made on the fate of toxin in contact with tissues of susceptible and resistant species.

MATERIAL AND METHODS.

Animals. White mice, rats and guinea-pigs bred at the Institute.

Toxins and antitoxic sera. Three batches of toxin supplied by the Commonwealth Serum Laboratories, Royal Park, were used, and are referred to as toxin 1, 2 and 3 respectively. Standard diphtheria antitoxin was supplied by the U.S.A. Public Health Service.

Definition of unit. All estimations of diphtheria toxin were made by intradermal injections into guinea-pigs. The standard unit of diphtheria toxin was taken as that quantity of toxin which, when injected in a volume of 0·1 ml. intradermally into a guinea-pig, caused no reaction or occasionally a faint reddish colouration in 24 hours, but which, after 48 hours, caused a slight oedema with well-marked induration and redness in an area of 1 cm. diameter.

The readings were made by comparison with standard controls made by injection of known amounts of toxin in the same guinea-pig in which titration of the unknown was carried out.

Determination of fatal dose. The fatal dose of diphtheria toxin was determined on guinea pigs weighing 250 gm. (± 10 gm.) and on mice weighing 20 gm. (± 0·5 gm.).

Determinations were made by (i) intraperitoneal injections of different amounts of toxin in a volume of 1 ml. in the guinea-pig and mouse; (ii) intravenous injections, the toxin being injected into the tail vein of the mouse or the subcutaneous vein in the leg of the guinea-pig in a volume of 0·2 ml.; (iii) intracerebral injections which were performed by making a short incision penetrating to the parietal bone over the right cerebral hemisphere: the bone was perforated with a fine drill, and the toxin injected in a volume of 0·03 ml. through a needle 0·5 mm. in diameter.

Estimation of toxin in urine. Guinea-pigs and groups of five mice which had been injected intraperitoneally with toxin in amounts proportional to their weight, were placed in metabolism cages, and the urine was collected in a vessel containing 0·1 ml. of an M/2 phosphate buffer (Sörensen) of pH 7·0 with 1 p.c. merthiolate. The collected urine was spun at 3,000 revs. per minute and titrated.

Estimation of toxin in organs and skin. Preliminary experiments indicated that the best method for the extraction of toxin was to mince the tissues as finely as possible with scissors, adding to the mince the required amount of physiological saline, then freezing and thawing repeatedly. After centrifugation, the toxin could be titrated by the standard method. Control experiments in which known amounts of toxin were added to organ or skin extracts showed that the tissue substances in the extract did not interfere with the titration.

RESULTS.

Relationship of fatal dose to route of administration in guinea-pigs. The results of these experiments are set out in Table 1. The fatal dose for guinea-pigs differs considerably according to the route of administration, the differences in susceptibility being most marked when the results of intraperitoneal and intracerebral administration are compared. The ratios between the doses fatal after intracerebral, intravenous and intraperitoneal injections, respectively, were 1 : >3 : 7. The characteristic post-mortem findings of diphtheria intoxication in guinea-pigs were found in those injected intraperitoneally. In contrast to this, no obvious change of any kind could be observed by the naked eye in the guinea-pigs dying after intracerebral injection. The only post-mortem finding evident macroscopically was a small reddish nodule at the point of injection which was probably due to local trauma.

TABLE 1.

The results of injection of graded doses of diphtheria toxin by three routes in groups of three guinea-pigs.

Toxin 2.

Volume.	Units of toxin.	Route of injection.		Result.
1 ml.	350	intraperitoneal	a.	dead after 2 days
„	250	„	b.	„ „ 4 „
„	150	„	c.	survived 7 „
0·2 ml.	250	intravenous	a.	dead after 2 days
„	165	„	b.	„ „ 2 „
„	125	„	c.	„ „ 2 „
0·03 ml.	64	intracerebral	a.	dead after 24 hrs.
„	32	„	b.	„ „ 5 days
„	16	„	c.	„ „ 14 „

Relationship of fatal dose to route of administration in mice. The results of these experiments are set out in Table 2. It was observed that the dose fatal for mice was approximately the same after intravenous and intraperitoneal administration. If differences between intraperitoneal and intravenous fatal doses for mice exist at all they must be less marked than in guinea-pigs. The ratio between the intracerebral and the intraperitoneal fatal dose for mice is of the same order as the corresponding ratio in guinea-pigs. The ratio between the intracerebral, the intravenous and the intraperitoneal fatal dose in mice is 1: 7: 7.

TABLE 2.

The results of injection of graded doses of diphtheria toxin by three routes in mice.

Toxin 2.

Volume.	No. of units.	Route of injection.	Animal.	Result.
1 ml.	106,000	intraperitoneal	4 mice	3 dead after 48 hours; 1 severely ill, killed.
„	21,200	„	4 „	2 „ „ 5 days; 2 dead after 7 days.
„	10,000	„	4 „	1 „ „ 5 „ ; 1 „ „ 7 „ ; 2 survived.
1 ml.	106,000	intraperitoneal	4 mice	4 dead after 48 hours.
„	10,600	„	4 „	2 „ „ 6 days; 2 survived.
„	2,120	„	4 „	4 survived.
0·5 ml.	53,000	intravenous	1 mouse	1 dead after 2 days.
0·4 ml.	42,400	„	1 „	1 „ „ 2 „
0·3 ml.	31,800	„	1 „	1 „ „ 2 „
0·2 ml.	21,200	„	1 „	1 „ „ 6 „
0·15 ml.	15,900	„	2 mice	2 „ „ 6 „
0·1 ml.	10,600	„	1 mouse	1 survived.
0·03 ml.	3,180	intracerebral	2 mice	2 dead after 5 days.
0·03 ml.	3,180	„	2 „	1 „ „ 4 „ ; 1 survived 7 days.
0·03 ml.	1,262	„	2 „	1 „ „ 7 „ ; 1 „ „ 7 „
0·03 ml.	636	„	2 „	1 „ „ 7 „ ; 1 „
0·03 ml.	636	„	2 „	2 survived.
0·03 ml.	3·8	„	2 „	2 „

The only pathological finding regularly observed in mice dying from diphtheria intoxication was dilatation of the small intestines. Occasionally paleness of the adrenals, partial consolidation of the lungs and parenchymatous degeneration of kidney or liver were noted, and in one mouse of the six injected intravenously marked hyperaemia of one adrenal was observed.

Comparison of fatal doses for guinea-pigs and mice. The ratios between the susceptibility of mice and guinea-pigs to diphtheria toxin are set out in Table 3.

TABLE 3.

Relationship of fatal dose of diphtheria toxin to route of administration in mice and guinea-pigs.

Toxin 2.

Doses killing mice and guinea-pigs within 4–6 days.

	No of units for guinea-pig.	No of units for mouse.	Ratio.
Intraperitoneal	250	21,200	1/85
Intravenous	< 125	21,200	> 1/170
Intracerebral	32	3,200	1/100

The opinion has been expressed in the literature that the resistance of rats and mice to diphtheria toxin is only a partial resistance in that their susceptibility to intracerebral administration is rather high. This produces an erroneous impression; the only statement which is permissible is that the central nervous system is 7 times more susceptible to diphtheria toxin, no difference at all existing between the resistant and the susceptible species in this respect.

Comparison of the sensitivity of the skin of mice and guinea-pigs to diphtheria toxin. Toxin I produced a marked reaction in the guinea-pig when 0·1 ml. of a 1/4,000 dilution was injected intradermally. The minimal reactive dose in the guinea-pig's skin was, therefore, of the order of 0·000025 ml. In mice no reaction was caused by injection of 0·05 ml. of the undiluted toxin in the skin either of the abdomen or of the ear.

TABLE 4.

Comparison of the amount of diphtheria toxin excreted in the urine of guinea-pigs and mice.

Toxin 2.

	Guinea-pig.				Titration of toxin in urine.				Group of 5 mice.				Titration of toxin in urine.			
Exp. No.	Wt. of animal.	No. of units toxin injected.	Time elapsing after injection.	Volume of urine collected.	Dilution of urine urine injected.	Intradermal reaction.	No. of units toxin estimated.	Total number of units excreted.	Wt. of animal.	No. of units toxin injected.	Time elapsing after injection.	Volume of urine collected.	Dilution of urine urine injected.	Intradermal reaction.	No. of units toxin estimated.	Total number of units excreted.
	gm.		hrs.	ml.					gm.		hrs.	ml.				
1	228	35,300	2½	1·1	1/5	—			87	13,680	2½	2·2	1/15	—		
					1/2·5	+							1/10	—		
					undil.	++	27						1/5	sl.	55	
			4½	3·4	1/5	—					4½	1·8	1/7·5	—		
					1/2·5	—							1/2·5	+		
					undil.	+	34						undil.	++	45	
			24	4·5	1/5	—					24	4·7	1/2·5	±		
					1/2·5	+							undil.	±	24	
					undil.	++	110	171								124
	Guinea-pig died after 30 hours.															
2	275	11,130	6	10	1/5	—			131	5,300	6	14	1/5	—		
					1/2·5	—							1/2·5	—		
					undil.	±	50						undil.	±	70	
			24	11	1/5	±					24	4·7	1/5	—		
					1/2·5	+							1/2·5	—		
					undil.	++	410	460					undil.	—	—	70
3	230	35,510	6	4	1/5	±			114	17,670	6	3·9	1/5	—		
					1/2·5	+							1/2·5	±		
					undil.	++	100						undil.	++	66	
			24	19·3	1/5	±					24	3·5	1/5	—		
					1/2·5	++							1/2·5	—		
					undil.	++	740	840					undil.	—	—	66
										53,000	6	4·5	1/5	—		
													1/2·5	±		
													undil.	++	68	
											24		1/5	—		
													1/2·5	—		
													undil.	—	—	68

TABLE 5.

Percentage of injected toxin excreted in urine of guinea-pigs and mice.

Toxin 2.

Time elapsed after injection. hours.	Guinea-pigs. P.c. of toxin excreted.	Total p.c. excreted.	Mice. P.c. of toxin excreted.	Total p.c. excreted.
Experiment 1.				
2½	0·08		0·4	
2½–4½	0·10		0·33	
4½–24	0·32	0·49	0·24	0·9
Experiment 2.				
6	0·5		1·3	
6–24	3·6	4·1	—	1·3
Experiment 3.				
6	0·28		0·37	
6–24	2·08	2·4	—	0·37

The difference in the resistance of the skin of guinea pigs and mice to the local action of diphtheria toxin is, therefore, of a much higher order than the difference in the lethal action of the toxin. The ratio between the doses of toxin fatal for guinea-pig and mouse is roughly of the order 1: 100, whereas the ratio between those producing a skin reaction is of the order of 1: 20,000. The central nervous system of the susceptible guinea-pig thus is shown to have the same higher degree of susceptibility than the systemic tissues as occurs in the mouse. In contradistinction to this, the resistance of the skin of the mouse is of an incomparably higher order.

Excretion of diphtheria toxin in urine of mice and guinea-pigs. It has been found that, when guinea-pigs and mice are injected with diphtheria toxin, portion is excreted in the urine unchanged (Bieling and Gottschalk, 1923; Pettit, 1914). A re-examination of the problem on a quantitative basis was thought to be the best means of deciding whether the rapid excretion of the toxin contributed to the natural resistance of the mouse.

Excretion of toxin was estimated in parallel experiments on guinea-pigs and mice which were injected intraperitoneally with the same number of units per gm. of body weight. The results are set out in Tables 4 and 5. In expressing the results of the estimation of toxin present a positive intradermal reaction corresponding to one unit is designated "+", of more than one unit "++", and less than one unit "±".

Even allowing for the usual experimental variations it is quite clear that, when given the same number of units of toxin per gm. of body weight, mice excrete less toxin than guinea-pigs. Renal excretion of part of the injected diphtheria toxin in an unaltered condition, therefore, does not appear to play an important part in the mechanism of the natural resistance of the mouse. As the fraction of injected toxin excreted is small, amounts of toxin less than the fatal dose for guinea-pigs could not be used, thus the objection could be made that the amount of toxin injected into the guinea-pigs was of such magnitude as to cause interference with the normal excretory mechanism. It seems most probable, however, that any interference with the normal excretory mechanism would become apparent by cessation of toxin excretion. Even if this had been the case, the fact remains that the mouse, without succumbing to the effect of the toxin, retains a larger proportion than does the guinea-pig which dies. This strongly suggests that the mouse has a greater ability to destroy toxin than has the guinea-pig.

The observation that the highest excretion of toxin by mice occurs within the first few hours after injection of toxin, whereas the guinea-pig excretes toxin up to the time of death, lends additional support to this opinion.

Distribution of injected diphtheria toxin in the body of guinea-pigs and mice. Preliminary experiments indicated that only amounts of toxin amounting to 10 units per gm. of body weight could be traced in the tissues with any accuracy.

In tests performed with different organs taken from guinea-pigs and mice at various times after the injection of toxin, it was found that toxin was invariably present in the blood, liver, spleen, kidney and adrenals. None could be found in the brain, heart or skeletal muscle. The titration experiments were, therefore, simplified and only blood, liver, spleen and kidney were taken for determination of toxin content. The technique of the titration of toxin present in organs has been described in "Material and Methods". The organ pulp had, of course, to be mixed with equal parts of physiological saline. The dilutions referred to in the Table as 1: 2 correspond to tests performed with the undiluted supernatant fluid. For other tests the supernatant was diluted 1: 3, which corresponds to a dilution of the organ 1: 6.

In Tables 6 and 7 the toxin contents of the various organs of guinea-pigs and mice following intravenous and subcutaneous injections are given. The Tables are set out in the manner used to record the results of the experiments on toxin excretion.

These results show that there exists no marked difference in the distribution of demonstrable toxin in guinea-pigs and mice. In both species some of the toxin is absorbed by liver, spleen and kidney within the first ninety minutes after injection, but most can still be found in the serum. As accurate toxin titrations in whole blood are impossible owing to the marked discolouration of the skin which follows injection, nothing definite can be said regarding the relative toxin content of the fluid and cellular elements of the whole blood. Should all the toxin present in the blood be in the serum, then the toxin content of the blood per ml. would be of the same order as that of the organs per gm., 90 minutes after the injection of the test dose. Four hours after injection the organs are usually free of toxin. Only occasionally was toxin found in the spleen and kidneys of mice, whereas the organs of guinea-pigs were always free. In this connection it might be mentioned that no free toxin could be found in the organs of guinea-pigs recently dead from diphtheria intoxication.

The only conclusions which may be drawn from the facts reported are that part of the injected toxin is quickly absorbed by the parenchymatous organs and that this absorbed toxin, in a short time, either becomes so firmly bound that it cannot be extracted, or else is completely destroyed by the organs.

A fall in the toxin content of the serum was always found after 4 hours; therefore, the toxin, which could be traced in the organs up to 1½ hours after injection, cannot have been re-absorbed into the blood. The possibility might be considered that the toxin which was set free by the organs and re-absorbed into the blood stream is excreted so rapidly by the kidneys that no increase

TABLE 6.

Distribution of injected diphtheria toxin in the body of the guinea-pig.

Toxin 3.

Guinea-pig.

Time elapsing after injection.	Weight of animal.	No. of units injected.	Route of injection.	Liver.				Spleen.				Kidneys.				Serum.		
				Weight.	Dilution injected.	Intradermal reaction.	Toxin content per gm. of organ.	Weight.	Dilution injected.	Intradermal reaction.	Toxin content per gm. of organ.	Weight.	Dilution injected.	Intradermal reaction.	Toxin content per gm. of organ.	Dilution injected.	Intradermal reaction.	Toxin content per ml.
hrs.	gm.			gm.				gm.				gm.						
1½	270	2,700	intravenous	9	1/6	—		0·37	1/6	.		2·39	1/6	±		1/6	+	
					1/2	—	—		1/2	++	25		1/2	+	20	1/3	++	
																undil.	++	60
4	270	2,700	„	9	1/6	—		0·5	1/6	—		2·29	1/6	—		1/6	—	
					1/2	—	—		1/2	—	—		1/2	trace	—	1/3	±	
																undil.	++	20
1½	330	3,300	„	13	1/6	—		0·54	1/6	trace		3·25	1/6	trace		1/6	±	
					1/2	±	15		1/2	++	40		1/2	++	40	1/3	++	
																undil.	++	38
4	330	3,300	„	11·5	1/6	—		0·42	1/6	—		3·3	1/6	—		1/6	±	
					1/2	—	—		1/2	—	—		1/2	—	—	1/3	+	
																undil.	++	30
4	290	2,900	subcutaneous	10	1/6	—		0·47	1/6	—		2·74	1/6	—		1/6	±	
					1/2	—	—		1/2	—	—		1/2	—	—	1/3	+	
																undil.	++	30

TABLE 7.

Distribution of injected diphtheria toxin in the body of the mouse.

Toxin 3.

Mouse.

Time elapsing after injection.	Weight of animal.	No. of units injected.	Route of injection.	Liver.				Spleen.				Kidneys.				Serum.		
				Weight.	Dilution injected.	Intradermal reaction.	Toxin content per gm. of organ.	Weight.	Dilution injected.	Intradermal reaction.	Toxin content per gm. of organ.	Weight.	Dilution injected.	Intradermal reaction.	Toxin content per gm. of organ.	Dilution injected.	Intradermal reaction.	Toxin content per ml.
hrs.	gm.			gm.				gm.				gm.						
1½	25	250	intravenous	2·40	1/6	±		0·62	1/6	±		0·65	1/6	±		1/6	±	
					1/2	±	15		1/2	++	25		1/2	++	45	1/3	++	
																undil.	++	45
4	25	250	,,	1·92	1/6	—		0·55	1/6	±		0·62	1/6	—		1/6	±	
					1/2	—	—		1/2	+	20		1/2	trace	—	1/3	+	
																undil.	++	30
1½	22·3	223	intravenous	2·00	1/6	—		0·36	1/6	—		0·49	1/6	—		1/6	++	
					1/2	—	—		1/2	±	5		1/2	+	20	1/3	++	
																conc.	++	>60
4	22·3	223	,,	1·80	1/6	—		0·43	1/6	—		0·41	1/6	±		1/6	+	
					1/2	—	—		1/2	trace	—		1/2	++	30	1/3	++	
																undil.	++	60
1½	20	200	intravenous	2·24	1/6	±		0·71	1/6	±		0·46	1/6	.		1/6	+	
					1/2	±	15		1/2	++	30		1/2	++	40	1/3	++	
																undil.	++	60
4	20	200	,,	2·33	1/6	—		0·35	1/6	—		0·45	1/6	—		1/6	—	
					1/2	—	—		1/2	trace	—		1/2	trace	—	1/3	±	
																undil.	++	20
4	20	200	subcutaneous	1·9	1/6	—		0·29	1/6	.		0·38	1/6	.		1/6	±	
					1/2	trace	—		1/2	—	—		1/2	trace	—	1/3	+	
																undil.	++	30

in toxin content of the serum is demonstrable. In my experiments only relatively small amounts of toxin could be found in the urine during the first 3 hours after injection. The amount of toxin excreted in the urine cannot, therefore, account for the disappearance of toxin from the organs of the mouse and the guinea-pig. Bieling and Gottschalk (1923) found that 5 hours after injection of large amounts of tetanus toxin, no free toxin could be demonstrated in organ extracts, whereas, injection of organ pulp caused typical tetanus in test animals. Similar experiments with diphtheria toxin cannot be carried out owing the the impossibility of injecting organ pulp through an intradermal needle. As the distributions of tetanus toxin and of diphtheria toxin after injection into the animal body are nearly identical, it is probable that diphtheria toxin is so firmly bound that it cannot be extracted from the organs.

The impression gained from these experiments is that some special type of mechanism, which has a rather long latent period, is responsible for the binding or destruction of the toxin in the organs. Thus, the toxin absorbed by the organs can be demonstrated after 1½ hours, but not after longer periods. The objection which could be made, that the large amount of toxin injected is relatively more toxic in the case of the guinea-pig and destroys the ability of the organs to absorb more toxin from the blood stream, and that therefore no toxin can be demonstrated 4 hours after injection, cannot possibly hold in the case of the mouse because the amounts of toxin injected were quite small in comparison with the fatal dose.

Percentages of demonstrable and non-demonstrable toxin. Knowing the absolute amounts of toxin demonstrated in blood and organs and the number of units injected into the animals, it has been found that 40 p.c. of the injected toxin can actually be demonstrated in the organs of the mouse 90 minutes after injection, whereas only 20 p.c. of the injected toxin is demonstrable in the organs of the guinea-pig (see Table 8).

TABLE 8.

Percentage of injected toxin demonstrable in the body of guinea-pigs and mice.

Toxin 3.

Time interval after injection.	P.c. of toxin injected found in these organs				P.c. toxin injected which is accounted for.	P.c. toxin injected not accounted for.
	Liver.	Spleen.	Kidney.	Serum.		
Mice.						
1½ hours	14	2	7	17	42	58
4 „		1·4	2	11·5	15	85
Guinea-pigs.						
1½ hours	2·4	0·4	2·6	14·8	20·4	79·6
4 „				9·4	9·4	90·6

Brain and muscle are practically free of toxin. Toxin unaccounted for would, therefore, be in tissues such as bone, skin or bowel (the toxin content of which has not been estimated), destroyed, or so firmly bound that it escapes detection by the methods employed. Results indicate that the hypothetical mechanism which accounts for the disappearance of toxin is more active in the susceptible guinea-pig than in the resistant mouse.

Bieling and Gottschalk found that a low grade immunity in the guinea-pig causes a higher content of demonstrable toxin in the organs of this susceptible species than occurs in a non-immunized animal. In the case of the resistant mouse a high content of demonstrable toxin has been found thus establishing a certain parallelism between partial immunity and natural resistance.

It is impossible to decide with any degree of certainty what happens to the toxin after absorption by the organs, but modern physico-chemical conceptions of immunology such as outlined by Pauling (1940) make it very probable that the demonstrable toxin is adsorbed in the surface layer of the cell. It may, therefore, be assumed that toxin which is not demonstrable by extraction, but which can be demonstrated by injection of organ pulp, has penetrated beyond this layer into the interior of the cell. According to this hypothesis the cells of the susceptible guinea-pig are more permeable to the toxin than those of the resistant mouse.

Disappearance of intradermally injected diphtheria toxin. A series of experiments was carried out in which a known number of units of toxin was injected intradermally into guinea-pigs and mice. After varying intervals the animals were killed, the portion of the skin in which the injection had been made was excised and its toxin content was estimated in the usual fashion. The results are set out in Table 9. It appears that the toxin disappears slightly more quickly from the skin of the mouse than from the skin of the guinea-pig. Many factors could contribute to this disappearance, therefore no far-reaching conclusions can be drawn. The high resistance of the skin of the mouse compared with that of the guinea-pig cannot be explained by this slight difference.

TABLE 9.

Results of estimation of toxin remaining in skin after intradermal injection of 20 units.

	Toxin 3.					
	Guinea-pig.			Mouse.		
Time elapsing after injection. hrs.	Dilution of toxin originally in skin.	Intradermal reaction.	Number of units of toxin remaining.	Dilution of toxin originally in skin.	Intradermal reaction.	Number of units of toxin remaining.
2	1/8	+		1/8	+	
	1/4	++	10	1/4	++	10
5	1/8	±		1/8	−	
	1/4	±	2	1/4	±	< 2
3	1/8	±		1/8	±	
	1/4	+	4	1/4	+	4
6	1/8	−		1/8	−	
	1/4	±	2	1/4	±	2
3	1/5	+	5	1/5	−	−
6	1/5	−	−	1/5	±	< 5
5	1/20	±		1/20	−	
	1/10	+		1/10	±	
	1/5	++	15	1/5	+	7·5

SUMMARY AND CONCLUSIONS.

The fatal doses of diphtheria toxin for mice and guinea-pigs have been determined. The ratios of the doses which were fatal for mice when administered by intracerebral, intravenous and intraperitoneal routes were $1 : > 3 : 7$. The corresponding ratios of fatal doses in the case of the guinea-pig were $1 : 7 : 7$.

Using various routes of administration, the doses of toxin fatal for mice have been compared with those fatal for guinea-pigs. The ratios found in the case of intraperitoneal, intravenous and intracerebral routes were 85 : 1, 170 : 1 and 100 : 1 respectively. The susceptibility of the mouse relative to that of the guinea-pig was, therefore, of the same order by both intraperitonal and intracerebral routes.

The sensitivity of the skin of the mouse as compared with that of the skin of the guinea-pig was of the order of 20,000 : 1. It was concluded that this was the only instance of a true variation in susceptibility of the tissues of these two species.

The relative amounts of diphtheria toxin excreted by the kidneys of mice and guinea-pigs injected with the same number of units of toxin per gm. of body weight were investigated. Under the conditions of the experiments guinea-pigs excreted a larger proportion of the injected toxin than did mice.

The toxin content of the organs of mice and guinea-pigs after injection of a known number of units of toxin has been studied. Ninety minutes after injection 40 p.c. of the toxin could be traced in the mouse as compared with 20 p.c. in the guinea-pig.

The rates of disappearance of intradermally injected toxin from the skin of mice and guinea-pigs respectively were determined. The differences found were too slight to afford an explanation of the exceptionally high resistance of the skin of the mouse.

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THE USE OF FORMALDEHYDE AND 2,6-DICHLORPHENOL-INDOPHENOL IN THE ESTIMATION OF ASCORBIC ACID AND DEHYDROASCORBIC ACID

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(Accepted for publication 27th October, 1942.)

INTRODUCTION.

Chemical methods of estimating the ascorbic acid and dehydroascorbic acid contents of biological materials customarily involve a selective extraction with an aqueous solvent (if the substances are not already in solution) and an assay of the extract; and it is usually assumed that the substances exist entirely in the free state in the materials and that they are completely extractable.

In those methods which depend ultimately upon the reducing nature of ascorbic acid (and almost all the procedures in current use are of this type) the presence of other reducing substances constitutes a potential source of error. Among extraneous reducers which may be present, there have been recognized: sulphhydryl compounds, ferrous compounds, substances formed by the decomposition of sugars in warm alkaline solution, phenols, polyphenols, sulphites and thiosulphates (see for example: Mottern, Nelson and Walker, 1932; Medes, 1935; Emmerie, 1934; van Eekelen and Emmerie, 1936; Birch, Harris and Ray, 1933; Basu and Nath, 1938; Lorenz and Arnold, 1938).

Methods which depend upon other properties and reactions of ascorbic acid, such as absorption of radiation at 265$\mu\mu$ (Robertson, 1934), decomposition to furfural followed by estimation of the furfural (Roe, 1936) and precipitation (of dehydroascorbic acid) by 2, 4–dinitrophenylhydrazine (Roe and Keuther, 1942), may avoid troubles due to the presence of extraneous reducers, and introduce new sources of error. At all events, they have as yet had only a limited application.

At the present time the only widely used methods depend upon reduction of the dye, 2, 6–dichlorphenolindophenol, measured titrimetrically (e.g. Tillmans, Hirsch and Jackisch, 1932; Bessey and King, 1933) or colorimetrically (e.g. Mindlin and Butler, 1937–8; Evelyn, Malloy and Rosen, 1938; Bessey, 1938), and despite its known shortcomings, this basis of procedure seems to be the most reliable yet established.

The work described in this article was done in preparation for an examination of germinating seeds as sources of ascorbic and dehydroascorbic acids.

Although Reid (1938) could not detect sulphhydryl compounds in extracts of germinating seeds of Vigna sinensis, other investigators have referred to the presence of these substances in germinating legume seeds (e.g. Harris and Ray, 1933; Johnson, 1933; Ray, 1934), the last-named restricting his ascorbic acid estimations to the axial organs because he considered that the high concentration of sulphhydryl compounds in the cotyledon extracts would hopelessly vitiate the titrations with dye solution.

In the light of this earlier work with germinating seeds, it was desirable to find a procedure in which the interference of sulphhydryl compounds would be minimized. A further incentive was derived from the fact that the reduction of dehydroascorbic acid to ascorbic acid, as one of the steps in its estimation, is usually done by treating extracts with H_2S; and the H_2S is known to react with certain quinones, aldehydes, keto-acids, etc. (Smythe and King, 1942), to form powerful reducing substances, some of which may be of a sulphhydryl nature (King, 1941).

The simpler modifications of the titrimetric and colorimetric procedures are to titrate in acid solution to a point at which the coloration due to a drop of dye solution will remain visible

for only several seconds, and to estimate the rate as well as the extent of reduction when using the photoelectric colorimeter and extrapolate back to almost zero time (Evelyn *et al*, 1938; Bessey, 1938). The assumption underlying these modifications is that the only rapidly reducing substance present is ascorbic acid.

Emmerie and van Eekelen (1934) removed certain sulphhydryl compounds from extracts by precipitation with mercuric acetate; but as King (1941) has pointed out, the extracts require subsequent treatment with H_2S and a fresh source of error is introduced.

Mason (1930) and Lugg (1932) showed that the reducing nature of cysteine could be greatly diminished by prior treatment with formaldehyde and mercuric chloride respectively. Medes (1935) employed these reactions in a colorimetric estimation of ascorbic acid with phospho–18–tungstic acid at pH 5, but observed that formaldehyde decreased the rate of reduction by ascorbic acid (see also Krishnamurthy, 1941) and that in the absence of sulphite, mercuric chloride appeared to oxidize the ascorbic acid.

In preliminary experiments, using a rough titrimetric procedure, it was found that over the pH range 1·5–3·5, $CdCl_2$ (Lugg, 1932) scarcely affected the rate at which cysteine reduced the dye. $HgCl_2$ was effective in diminishing the rate, but it reacted fairly rapidly with ascorbic acid giving a white precipitate (Hg_2Cl_2 ?) and diminishing the reduction by the ascorbic acid (cf. Medes 1935). Formaldehyde greatly diminished the rate of reduction by cysteine. At pH 3·5 it reacted fairly rapidly with ascorbic acid to diminish the reduction by ascorbic acid, but at pH 1·5 its reaction with ascorbic acid was slow. It was found also that formaldehyde greatly diminished reduction of the dye by sulphites, thiosulphates and H_2S over the pH range, thus securing the same effect with sulphites as do acetone (Mapson, 1941) and certain sugars (Kirkpatrick, 1941). Attention thereafter was focussed upon the exploitation of the reactions with formaldehyde.

EXPERIMENTS AND DISCUSSION.

The crude titrimetric procedure was considerably refined. The 10 ml. aliquots of solution taken for titration normally contained metaphosphoric and pyrophosphoric acids. Since the introduction of solutions of the former (Fujita and Iwatake, 1935) and the latter (Giri, 1937–8) as extractants, evidence has accumulated that they greatly retard catalytic oxidation of the ascorbic acid by enzymes and heavy metals.

These acids were included in the form of technical "metaphosphoric" acid, a glassy material containing 18 p.c. Na_2O, 32 p.c. P_2O_5 as metaphosphate, 35 p.c. P_2O_5 as pyrophosphate, 4 p.c. P_2O_5 as orthophosphate, and 9 p.c. H_2O in combination.[1]

The degree of polymerization of the HPO_3 (see Mellor, 1928) was unknown. For convenience the material will be referred to as "metaphosphoric" acid. The solutions were adjusted to various pH values lying between 1·5 approx. and 3·5 approx. Solutions at pH 3·5 contained in 10 ml. about 0·26 gm. of "metaphosphoric" acid, 0·21 gm. crystalline citric acid ($C_6H_8O_7$. H_2O) and 0·1 gm. NaOH, whilst those at pH 1·5 contained about 0·065 gm. HCl in addition.

The 2, 6–dichlorphenolindophenol solution was run in from a 10 ml. burette with tip drawn out to deliver drops of 0·020 ml. The dye solution contained per ml. the equivalent of 0·5 mg. of the pure, acid dye (but in the form of the blue sodium salt) and each drop therefore contained 10γ.

By trial it was found that the red colouration due to 1γ of the dye in about 10 ml. of solution (pH < 4) was just perceptible to the eye under ordinary diffused daylight lighting in the laboratory, the solution being held in a flat-bottomed cylindrical vessel of colourless glass (internal diameter about 2·5 cm.) and viewed against a white background. A very satisfactory titration vessel is made by attaching twin bottles of suitable dimensions (weighing bottles, 2·7 cm. internal and 3·0 cm. external diameter and 6·0 cm. deep, have proved suitable) side by side to a narrow separating block placed up near the mouths of the bottles. One of the bottles holds test solution for titration, and the other one some of the same solution as a comparison blank.[2] The time taken for the colouration due to one drop of dye (i.e. 10γ) solution, to fade to bare perceptibility (10 p.c. of its original value), was taken as the "persistence" (or strictly, the "period for 10 p.c. persistence").

The general procedure was to run the dye solution in with vigorous shaking and at the rate of one drop per 2 seconds until a "persistence" of about 2 seconds was reached. Thereafter successive drops were run in with careful timing of the "persistence" for each drop, and in some cases the titration was continued to a genuine "end-point", i.e. to the point at which about 10 p.c. of the colouration due to a drop would persist indefinitely (as far as the reducer was concerned). From the results it was possible to construct reasonably reproducible "drop-'persistence' titration curves". Most of the work was done at 25° C., but some study was made of the effects of variation in temperature.

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[1] According to analyses by Mr. I. Brown.

[2] The most satisfactory comparison blank, especially when coloured products are formed during the titration, is made by adding to the blank an amount of dye solution just less than that required for the titration itself.

Consider the second order reversible reaction in which one dye molecule becomes a molecule of leucobase by acquiring two hydrogen atoms from a reducing substance: dye + $S_{red} \rightleftharpoons$ leucobase + S_{ox}.

Neglecting the fact that certain ionic species of the substances may be the real reactants, and using brackets to signify concentrations (approximating to the activities) we have equations (1), (2) and (3) expressing the forward velocity (V_α), the backward velocity (V_β), and the equilibrium, respectively; k_α, k_β and K being constants. The constants may vary, however, with pH, salt concentrations, etc.

$$k_\alpha [\text{dye}] [S_{red}] = V_\alpha \quad . \quad . \quad . \quad (1)$$

$$k_\beta [\text{leuco}] [S_{ox}] = V_\beta \quad . \quad . \quad . \quad (2)$$

$$\frac{[\text{leuco}] [S_{ox}]}{[\text{dye}] [S_{red}]} = \frac{k_\alpha}{k_\beta} = K \quad . \quad . \quad . \quad (3)$$

Ascorbic acid. k_α is fairly large and k_β very small. K is very large, in accordance with the known E_0' values[3] of the leucobase: dye system (+ 0·58 volt at pH 1·5, + 0·46 volt at pH 3·5, extrapolated from the 30° C. data of Gibbs, Cohen and Cannan, 1925) and of the ascorbic acid: dehydroascorbic acid system (+ 0·30 volt at pH 1·5, + 0·18 volt at pH 3·5, interpolated from the 30° C. data of Ball, 1937). Consider the beginning of a titration. Ignoring catalytic effects, if any, we have of the dye introduced in the first drop: (say) 50 p.c. persistence after x seconds, 25 p.c. after 2x sec., 12·5 p.c. after 3x sec., 6·2 p.c. after 4x sec., and so on, the p.c. persistence asymptotically approaching, not zero, but the very small positive value required by equation (3). As successive drops are added x becomes larger. At half titration it is about twice as large as at the beginning provided the volume has not been increased unduly, but even when only a small fraction of the ascorbic acid is left, x is still fairly small. When only about 0·02 mg. ascorbic acid is left, x is still less than 1 at 25° C., 90 p.c. of the dye in the last drop having been reduced in about 3 sec. With three more drops a faint "end-point" of considerable stability is reached. In this case, where k_α is fairly large and K is very large, we have a reasonably "sharp" and almost "complete" titration. Examples are seen in curves $3A_0$, Fig. 1 and $3A_0$, Fig. 2.

Quinol (1, 4–benzoquinol). k_β is greater than k_α, but both are fairly large. The titration is fairly "sharp" but is very "incomplete". After a few drops of dye solution have been added a faint but highly stable "end-point" is reached. Further addition of dye solution promptly, but only slightly, increases the colouration of the "end-point". These phenomena are in accordance with the fact that the E_0' values of the quinol: quinone system (+ 0·62 volt at pH 1·5, + 0·50 volt at pH 3·5, from 30° C. data reviewed by Clark, 1928) are actually a little more positive than those of the leucobase: dye system at corresponding pH values. Examples are seen in curves $14A_0$, Fig. 1 and $14A_0$, Fig. 2.

These two cases have been selected for description, because they may both be treated in terms of equations (1), (2) and (3), because the stoichiometry of the reactions is known or may be inferred, because they illustrate two very different types of actual titrations, and because the descriptions of phenomena apply over the pH range, 1·5–3·5. In the remaining cases to be considered in detail the order of the reactions may be different, or the stoichiometry may be obscure, or the effects of change of pH may be pronounced, or there may be other factors which complicate the description.

Sulphite ($Na_2S_2O_5$). The titration is "sluggish" at pH 1·5 and pH 3·5. Examples are seen in curves $7A_0$, Fig. 1 and $7A_0$, Fig. 2. The stoichiometry, which does not correspond with oxidation to sulphate, has not been investigated thoroughly.

Thiosulphate ($Na_2S_2O_3$, $5H_2O$). k_α is fairly large at pH 1·5, but fairly small at pH 3·5 (cf. curves $6A_0$, Fig. 1 and $6A_0$, Fig. 2). The stoichiometry of the reaction is obscure, the degree of oxidation for highly stable "end-points" being about the same at pH 1·5 and pH 3·5 and corresponding with the addition of 3 oxygen atoms to 4 molecules of $Na_2S_2O_3$.

H_2S (Na_2S, $9H_2O$). The stoichiometry is obscure because a side-reaction leads to the early establishment of a false "end-point". The colouration, unlike that due to the presence of the dye, is not discharged by addition of ascorbic acid. For this reason the titrations, which are quite "sluggish", were not carried far.

Ferrous salts. In the case of $FeSO_4,(NH_4)_2SO_4,6H_2O$ in HCl solution at pH 1–3, there is virtually no reduction of the dye. This is in accordance with the fact that the E_0' value of the Fe++: Fe+++ system (+ 0·74 volt at pH 1·5 and pH 3·5), is distinctly positive to those of the leucobase: dye system at these pH values. Anything which preferentially removes Fe+++ ions from the system favours reduction of the dye.

Thus the addition of large amounts of citric acid permits a very "sluggish" titration to be made because the Fe+++ ions enter into the formation of a complex, and Lorenz and Arnold (1938) have shown that the titration can be carried out in the presence of oxalic acid or "metaphosphoric" acid. In the presence of "metaphosphoric" acid, ferrous salts become extremely

[3] By E_0' is meant the potential of the 50 p.c. oxidized system at the stated pH value, relative to that of the normal hydrogen electrode.

vigorous reducers and readily react with dissolved oxygen. At pH 3·5 the rate of aerial oxidation is very much greater than at pH 1·5. On the other hand the rate of reaction with the dye is very much greater at pH 1·5 than at pH 3·5 (cf. curve $17A_0$, Fig. 1, with curve $17A_0$, Fig. 2), and if proper precautions are taken against aerial oxidation, the titration at pH 1·5 is extremely "sharp" and virtually "complete" and provides an excellent means of standardizing the dye solution.

Cysteine (cysteine hydrochloride). The main primary reaction with the dye is presumably a termolecular one involving the formation of cystine. At all events cystine does not appreciably reduce the dye over the pH range 1·5–3·5. But a side reaction in the titration of cysteine leads to the formation of a greenish-yellow colouration and the titrations, which are rather "sluggish", were not carried very far. Examples are seen in curves $11A_0$ and $12A_0$, Fig. 1, and curves $11A_0$ and $12A_0$, Fig. 2.

Contrary to what the literature had suggested, the curves at pH 1·5 and pH 3·5 appear to be very similar.

H_2S-treated pyruvic acid. Pyruvic acid itself was found to be almost without effect upon the dye over the pH range 1·5–3·5 (Curves $20A_0$, Fig. 1 and $20A_0$, Fig. 2). By maintaining a pressure of 1 atmosphere of H_2S over a solution of the substance at pH 3·5 for two hours at 25° C. and then removing H_2S by bubbling N_2 through for 45 minutes, a solution was obtained which smelled strongly of unidentified sulphur compounds. It readily reduced the dye over the pH range, but gave a pale yellow colouration, and special comparison blanks had to be used in the titrations. Titrations are represented by curves $20B_0$, Fig. 1, and $20B_0$, Fig. 2, and it will be seen that the material is more strongly reducing at the higher pH value. From the shapes of the curves it is surmised that the titration is a complex one at either pH value, involving a rapid and a relatively slow oxidation. The stoichiometry of the reactions is unknown.

Reductone. Gluco-reductone was made by heating 2 gm. glucose in about 90 ml. of 0·2 N NaOH solution at 75° C. for 30 min. After cooling, the dark brown solution was brought to approx. pH 2·5 by adding about 2 ml. of 5N HCl. The paler brown solution was diluted to 100 ml., decolourized considerably by addition of a little absorbent charcoal, and filtered from the charcoal. No attempt was made to separate and purify the reductone. The solution of impure reductone ("reductone") was used in the experiments. At pH 3·5 the titration was fairly "sharp", whilst at pH 1·5 it was rather "sluggish" (curves $21A_0$, Fig. 2, and $21A_0$, Fig. 1). Nothing is actually known of the stoichiometry of these titrations, though reductone itself may reduce in a way similar to ascorbic acid.

Effects of Formaldehyde.

It was found that formaldehyde, in the concentrations employed in the titrations, was without any definite effect upon the dye. This observation was confirmed later with the photoelectric colorimeter (cf. curve a_1 with a_2 and a_1' with a_2', in Fig. 5).

Formaldehyde is able to condense with a variety of reducing substances in such a way as to render them virtually non-reducing or feebly reducing. It must be stated that the only evidence for condensation in this work was an observable effect upon the reducing behaviour of the reducer concerned: other types of condensation may have occurred and passed unnoticed. The condensation reaction may be fast or slow, depending upon the particular reducer. Its velocity may also vary with pH. In some instances it was evident that the condensation reactions were strictly reversible. Titration of such mixtures is probably to be regarded as titration of the

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EXPLANATION OF FIGURES.

Figs. 1, 2, 3 and 4. (Drop-"persistence" titration curves at 25° C.)

Vol.: Approx. 10 ml. at beginning, and averaging 11·5 ml. at end of titration.

The letters signify respectively:

A, (no prior treatment with H_2S) : B, (prior treatment with H_2S).

The curve numbers signify respectively:

1, 2, 3, 4, (0·1, 0·25, 0·5, 1 mg. ascorbic acid) : 5, 6, (1, 2 mg. $Na_2S_2O_3$, $5H_2O$) : 7, 8, (1, 2 mg. $Na_2S_2O_5$) : 9, 10, (5, 10 mg. Na_2S, $9H_2O$) : 11, 12, 13, (1, 4, 16 mg. cysteine HCl) : 14, 15, (2, 8 mg. quinol) : 16, 17, 18, (0·5, 1, 2 mg. Mohr's Salt) : 19, 20, (16, 32 mg. pyruvic acid) : 21, 22, (0·2, 0·5 ml. of the "reductone" solution) : 23, (0·625 mg. 2–Me–1, 4–naphthaquinone) : 24, (5 mg. thiourea) : 25, (5 + 8 + 12 + 19B) : 26, (3 + 25) : 27, (extract from 1·60 gm. treated cabbage) : 28, (extract from 0·65 gm. germinating peas).

The inferior numbers signify respectively:

0, 1, (no "formalin") : 2, (0·2 ml. "formalin" for 5 min. at pH 1·5) : 3, (0·5 ml. "formalin" for 15 min. at pH 3·5, then brought to pH 1·5) : 4, (0·1 ml. "formalin" for 10 sec.) : 5, (0·1 ml. "formalin" for 10 min.) : 6, (0·5 ml. "formalin" for 10 sec.) : 7, (0·5 ml. "formalin" for 10 min.).

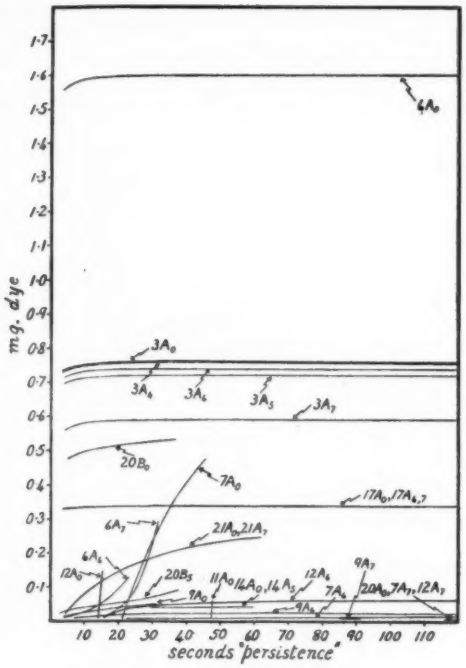

Fig. 1. Titrations at pH 1·5.

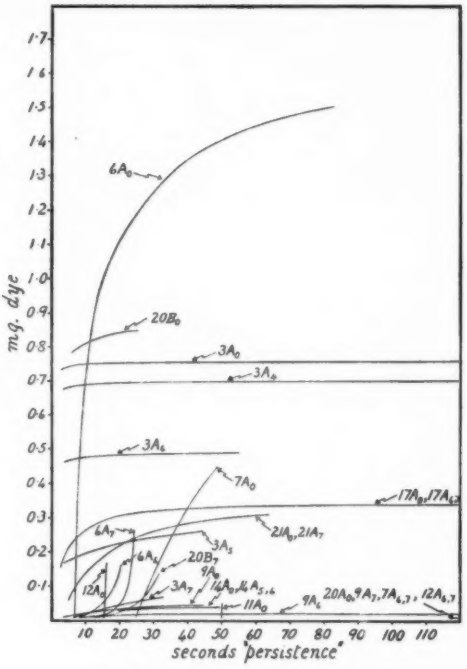

Fig. 2. Titrations at pH 3·5.

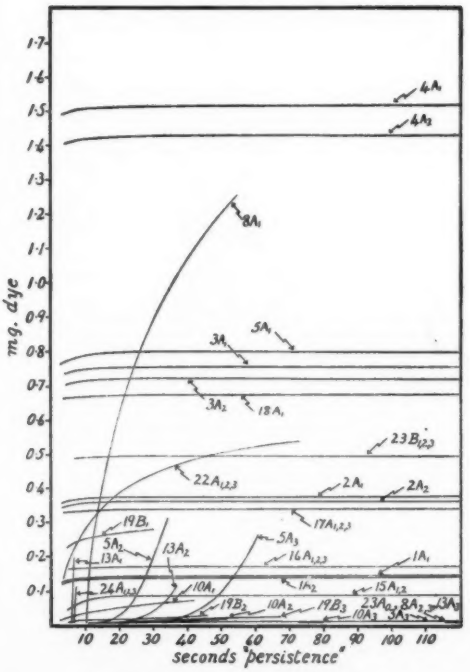

Fig. 3. Titrations at pH 1·5.

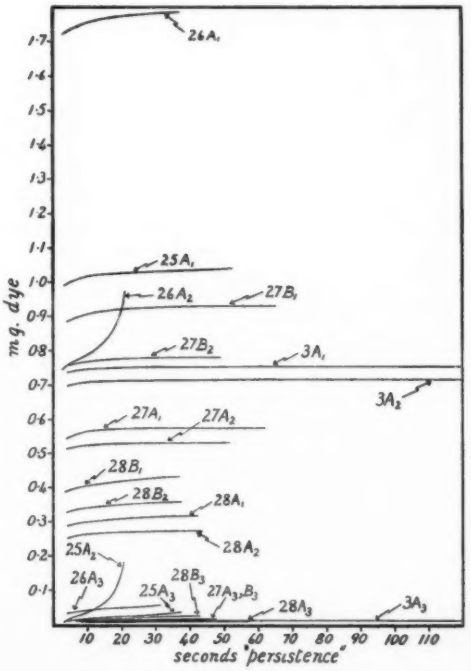

Fig. 4. Titrations at pH 1·5.

small amount of free reducing substance in the mixture. As it is oxidized away it is replenished by dissociation of the condensation product.

In making the drop-"persistence" titrations of mixtures which contained formaldehyde, the minimum practicable time (a few seconds) was allowed to elapse between a "fade-out" and the addition of the next drop of dye solution.

At pH 1·5 the velocity of condensation of ascorbic acid with formaldehyde was found to be very small. At pH 3·5 it was moderately large. The condensation equilibria at both pH values (under the conditions of these experiments) were greatly in favour of the condensation reaction. With 0·5 mg. and 1·0 mg. ascorbic acid, and 9 millimol. of HCHO at pH 3·5, there were about 0·01 mg. and 0·02 mg., respectively, of free ascorbic acid at equilibrium at 25° C. This was established by allowing the condensation to proceed almost to equilibrium, and then titrating with a little dye solution until the drop-"persistence" suddenly increased. On allowing the mixture to stand further for a considerable time the ascorbic acid obtained by dissociation of the condensation product could be titrated until, as before, the "persistence" suddenly increased. Means of such pairs of titration values served to establish approximately the equilibrium conditions. It must be mentioned that by bringing a mixture which has reacted at pH 3·5 to pH 1·5 by addition of acid, the system can be "fixed" as it were, for easier study, because both the condensation and dissociation reactions are enormously retarded by pH 1·5.

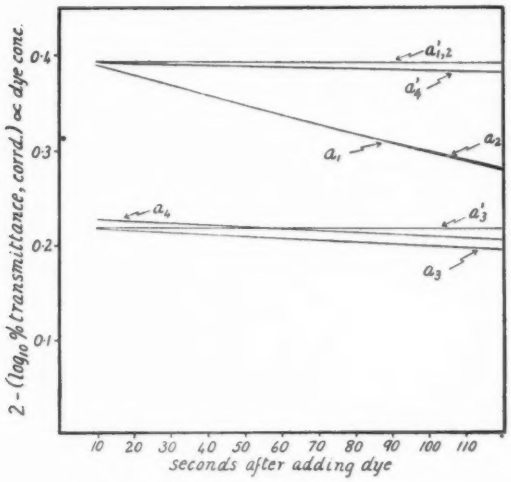

Fig. 5. Colorimetry at pH's 1·5 and 3·5.

Fig. 5. (Colorimeter curves of reduction and fading at 25° C.).

Vol.: 7·5 ml. before, and 8·0 ml. after adding dye solution. 0·138 mg. dye in each case, added last.

pH 1·5. a_1, (dye) : a_2, (0·15 ml. "formalin", dye) : a_3, (0·04 mg. ascorbic acid, dye) : a_4, (0·04 mg. ascorbic acid plus 0·15 ml. "formalin" for 5 min., dye).

pH 3·5. a_1', (dye) : a_2', (0·4 ml. "formalin", dye) : a_3', (0·04 mg. ascorbic acid, dye) : a_4', (0·04 mg. ascorbic acid plus 0·4 ml. 'formalin" for 10 min., dye).

These findings do not agree completely with Krishnamurthy's (1941) conclusions drawn from work done at pH values greater than 3·2, but his more acid reaction mixtures may not have come close to equilibrium conditions before analysis. His experiments show that the equilibria are approached rapidly at about pH 5 but are not overwhelmingly in favour of the condensation, a fact which is in accordance with Medes' (1935) use of formaldehyde.

Formaldehyde appeared to be without effect upon ferrous salts, quinol, and "reductone" at pH 1·5 or 3·5.

Even small amounts of formaldehyde condensed rapidly with cysteine, sulphite and thiosulphate, at pH 1·5 and 3·5, and the equilibria, under the conditions of most of the experiments, were greatly in favour of the condensation. The condensation product in the case of sulphite appears to be a hydroxy-sulphonic acid (Lauer and Langkammerer, 1935), and in the case of cysteine a substituted thiazolidine (Schubert, 1936).

Formaldehyde condensed rather less rapidly with H_2S and with H_2S-treated pyruvic acid. However, the velocities of condensation at pH 1·5 and 3·5 were by no means small.

Temperature Variation.

Most of the work was done at 25° C. as stated earlier. It was necessary to determine the effects of change of temperature upon the velocities and the equilibria of the more important reactions.

For the variation of velocity and equilibrium constants respectively with temperature, we have the empirical equation of Arrhenius (4) and the van't Hoff isochore (5).

$$d(\ln k)/dT = -E/RT^2 \quad . \quad . \quad . \quad (4)$$
$$d(\ln K)/dT = -U/RT^2 \quad . \quad . \quad . \quad (5)$$

where ln, T, R, E, U, k and K are natural logarithm, absolute temperature, the gas constant, energy of activation, total energy change, velocity constant and equilibrium constant, respectively.

Integrated with respect to T, over a range of temperatures ($T_1 - T_2$) for which E and U may be assumed constant, these equations become, respectively:

$$\ln(k_1/k_2) = E(T_2 - T_1)/RT_1T_2 \quad . \quad . \quad (6)$$
$$\ln(K_1/K_2) = U(T_2 - T_1)/RT_1T_2 \quad . \quad . \quad (7)$$

and from observed variations of k and K with T, E and U may then be calculated.

Drop-"persistence" titrations were made of ascorbic acid at pH 1·5 and at 10° C.–30° C. The volumes of dye solution required for highly stable "end-points" appeared to be identical. The equilibrium is overwhelmingly in favour of the forward reaction, and it was impossible by this method to detect any change in the equilibrium constant with temperature. The same titrations, however, yielded information on the temperature variation of the forward reaction velocity. Judged from the "persistence" of drops towards the end of the titrations, $k_{(T + 10°)}/k_{(T°)} = 1·5$, whence E = 7,000 cals.

In the main experiments concerned with determining the effects of change of temperature upon the condensation of formaldehyde with ascorbic acid, the pH was 3·5 and there was always an overwhelmingly large stoichiometric excess of formaldehyde (0·4–0·9 millimol. of formaldehyde per ml.). By measuring the velocity at varying concentrations of the reactants, it was established that the reaction was a simple bimolecular one. Titrations were made of the amounts of ascorbic acid remaining after condensation had proceeded for varying periods at temperatures ranging from 5° to 30°C. Some of the titrations were made at pH 3·5; others were made after bringing the reaction mixture to pH 1·5, where the rate of condensation is only 0·03 times the rate at pH 3·5. No definite variation of the equilibrium constant with temperature was revealed in these experiments.

The condensation velocity constant was found to vary with temperature in such a way that $k_{(T + 10°)}/k_{(T°)} = 3·0$, whence E = 18,700 cals.

No attempt was made to determine the effects of temperature variation upon the rates at which the other reducing substances reduce the dye or react with formaldehyde. As with most reactions the rates probably increase about twofold per 10° C. rise in temperature.

Aerial Oxidation.

It is well known that the rate of aerial oxidation of ascorbic acid is increased by the presence of certain Cu salts and by increase in pH value. According to Barron, DeMeio and Klemperer (1935–6), salts of Cu are ordinarily effective whilst those of Co, Mn, Ni, Fe, are not, and the catalytic action of Cu salts can be reduced by complex formation.

A few experiments were made with regard to the aerial oxidation of ascorbic acid in the presence of different amounts of Cu salts in "metaphosphoric" acid solution. The work was done at 25° C., the usual concentrations of "metaphosphoric" acid, citrate, etc., being used. Each 10 ml. of solution contained 0·2 mg. ascorbic acid. The conclusions drawn from this work were: In the presence of 1γ Cu per 10 ml. of solution there was no detectable oxidation of the ascorbic acid after aspirating air through the solution at pH 1·5 or pH 3·5 for one hour. On adding 100γ of Cu (as $CuSO_4,5H_2O$, concentrated solution) to a "metaphosphoric" acid solution of ascorbic acid at pH 3·5 the titration of the ascorbic acid was not affected, but if air were aspirated through the solution for 1 hr. there was a 21 p.c. loss of the titratable ascorbic acid. In contrast to these results is the oxidation of ferrous salt (1 mg. of Mohr's salt) in the "metaphosphoric" acid solution: the oxidation was virtually complete after 1 hour of aeration at pH 3·5 in the presence of 1γ of Cu, but the Cu was probably without effect.

METHODS OF ESTIMATION.

From the previous sections it will be clear that of the substances which ordinarily reduce the dye rapidly or fairly rapidly, some readily lose that property when allowed first to react with formaldehyde (Class 1, comprising cysteine, sulphite, H_2S-treated pyruvic acid, thiosulphate, etc.), others much less readily lose the property (Class 2, of which the only member known at present is ascorbic acid), and yet others appear to be unaffected by formaldehyde (Class 3, comprising quinol, "reductone", ferrous salts, etc.).

It should therefore be possible, by measuring (titrimetrically, colorimetrically, potentiometrically, etc.) the reduction of the dye under these varied conditions, not only to obtain a corrected estimate of the amount of ascorbic acid present, but, by noticing the rates of reduction generally as well as the effects due to formaldehyde treatment, to form some idea of the types and quantities of extraneous reducers present and thus, indirectly, some idea of the degree to which deficiencies of method may have permitted the estimation of ascorbic acid to be vitiated.

These considerations form the basis of the analytical procedures described below. At pH 1·5 the rate of decomposition of the dye is high (about 3 p.c. at 10° C. and 12 p.c. at 25° C., in 1 min.); and, a most troublesome feature, the rate is retarded if some of the dye has first been reduced by ascorbic acid (see Fig. 5). Consequently, although the photo-electric colorimeter can be used to measure reductions at this pH value, the errors of extrapolation to almost zero time may be large. The following procedure has been designed primarily to suit a drop-"persistence" titration technique.

Reagents.

Stock "metaphosphoric" acid solution. The solution contains 160 gm. of "metaphosphoric" acid per litre with, if necessary, sufficient HCl or NaOH added to bring the mixture (when diluted with water to about ⅕ the concentration) to pH 1·45–1·55. The "metaphosphoric" acid should be such that some 50 p.c. or more of the phosphorus in it is in the metaphosphoric acid condition and most of the remainder in the pyrophosphoric acid condition. The solution may be kept satisfactorily for a week at about 2°C.

Dilute "metaphosphoric" acid solution. A solution made by diluting one volume of the stock solution with water to four volumes.

Citrate buffer. A solution made by dissolving 52·5 gm. of crystalline citric acid ($C_6H_8O_7,H_2O$) and 24·0 gm. of NaOH in about 180 ml. of water with cooling, and diluting to 250 ml.

5N HCl solution. The concentration of HCl should be between 4·9 and 5·1N.

Formaldehyde solution. 18 M* aqueous solution (40 p.c. formaldehyde solution. "Formalin").

Stock dye solution. This is prepared by dissolving the equivalent of about 70 mg. of the pure acid dye, 2, 6–dichlorphenolindophenol (but as the blue sodium salt), in water containing 5 ml. of a pH 7·8 phosphate buffer (0·1 M with respect to phosphate radicles) and diluting to 100 ml. The solution may be kept for 3 or 4 days at about 2° C.

Standard dye solution. The stock dye solution is so diluted with water that 1 ml. contains the equivalent of 0·500 mg. of the free acid dye. The standardization is conducted against ascorbic acid, 1·52 ml. of dye solution being required to give a faint but highly stable "end-point" with 0·500 mg. of ascorbic acid dissolved in 10 ml. of the dilute "metaphosphoric" acid solution; but for greater convenience a routine standardization against ferrous salt is recommended.

Standard ferrous salt solution. 0·1000 gm. of Mohr's salt ($FeSO_4,(NH_4)_2SO_4,6H_2O$), dissolved in 0·1 N HCl solution (which has been saturated with nitrogen) and diluted to 100 ml. with more of the HCl solution. The solution oxidizes slowly in the air, and although the ferric salt may be reduced again it is recommended that the solution be made up freshly as required. Each ml. of the solution requires 0·682 ml. of the dye solution at the proper concentration. 1 or 2 ml. of the solution are added to 9 or 8 ml. of the dilute "metaphosphoric" acid reagent and the mixture is promptly titrated with the dye solution to a faint but highly stable "end-point".

PREPARATION OF EXTRACTS.

The provisional assumption is that the ascorbic and dehydroascorbic acids occur only in the readily diffusible, free state in the material under test. Prior heating with acid to destroy certain possible combinations has been advocated by Reedman and McHenry (1938), but for reasons to be given later the recommendation cannot be endorsed. The freeing of the substances from other possible combinations is also discussed later.

All reagents and water used in making up extracts (and standard solutions for the standardization of the dye solution) should be washed with nitrogen[4] before use, and all extracts and solutions prepared for titration should be washed with nitrogen and kept under nitrogen as much as possible during the analytical work (except in special circumstances to be described later).

[4] Carbon dioxide may be used instead of nitrogen throughout.

* *For* 18 M *read* 15 M.

The amount of test material taken should contain not more than 15 mg. and preferably not less than 1·5 mg. of "total" ascorbic acid (i.e. ascorbic acid plus dehydroascorbic acid calculated as ascorbic acid). The approximate water content of the material is noted. It is then ground in the presence of a little clean sand with some stock "metaphosphoric" acid reagent plus additional water (and, if necessary, some HCl or NaOH) to yield a mash of which the liquid portion will be of pH 1·55 and will contain "metaphosphoric" acid of ¼ the stock concentration, i.e. the same concentration as the dilute "metaphosphoric" acid reagent. If the original test material happens to be liquid instead of solid, the grinding is unnecessary.

The mash is centrifuged and the liquid is collected and washed with nitrogen. The residue is reground with some dilute "metaphosphoric" acid reagent and the mash is again centrifuged. The process is repeated as often as may be necessary to extract the bulk of the ascorbic and dehydroascorbic acids. Correction for the amounts retained by the moist residue can be calculated approximately from a simple "successive dilutions" formula. The combined extracts are diluted to 100 ml. with the dilute "metaphosphoric" acid reagent. Extreme clarity of extracts is seldom necessary.

ANALYSES OF EXTRACTS.

80 ml. of the solution are diluted with water to 100 ml. after sufficient of the citrate buffer (approx. 10 ml.) has been added to bring the final pH to 3·45–3·55. Of the resulting solution two 40 ml. aliquots are taken in 50 ml. standard flasks, A and B.

The contents of flask A are diluted with water to 50 ml. Three 10 ml. aliquots are taken for titration, A_1, A_2 and A_3. A_1 and A_2 are brought to pH 1·45–1·55 by addition of the 5N HCl reagent (approx. 0·35 ml.). A_1 is titrated directly. A_2 is treated with 0·20 ml. of the formaldehyde reagent for time t_1 (= 3 min. at 30° C.; 5 min. at 25° C.; 8·5 min. at 20° C.; 15 min. at 15° C.; 25 min. at 10° C.) before being titrated. Aliquot A_3 is allowed to react with 0·50 ml. of the formaldehyde reagent for time t_2 (= 9 min. at 30° C.; 15 min. at 25° C.; 25 min. at 20° C.; 45 min. at 15° C.; 75 min. at 10° C.) and is then brought to pH 1·45–1·55 by addition of the 5N HCl reagent (approx. 0·35 ml.) and the titration is begun within about 1 min.

The contents of flask B are saturated with H_2S by bubbling the gas through for about 15 min. and the flask is stoppered to enclose H_2S at one atmosphere pressure and allowed to stand for time t_3 (provisionally, 2 hours). It is believed that any dehydroascorbic acid present is almost completely reduced within 2 hrs. at 20° C. There may be special reasons (to be discussed later) for curtailing or prolonging the period of standing. Nitrogen is then blown through until excess H_2S has been almost completely removed (say 0·5 hr.) and the contents are diluted with water to 50 ml. Three 10 ml. aliquots, B_1, B_2 and B_3, are treated in a manner entirely analogous to those taken from flask A (A_1, A_2 and A_3, respectively).

For the best use of the data already presented, it is desirable to conduct the titrations as described in the Experimental section, with a burette delivering drops of 0·020 ml. of dye solution (each drop containing the equivalent of 0·01 mg. of the free dye and the periods for 10 p.c. persistence being recorded.

The drop-"persistence" titration curves are compared. In the absence of extraneous reducers, Curve A_1 will be about 5 p.c.[6] above A_2, and B_1 about 5 p.c.[6] above B_2, and curves A_3 and B_3 will lie almost on the baseline. In the presence of extraneous reducers like thiosulphate, sulphite, cysteine, etc., curve A_1 may be considerably above A_2, and B_1 considerably above B_2, and their shapes and slopes may be very different from those obtained with pure ascorbic acid; and in

[5] Addition of HCl or NaOH will seldom be necessary with biological materials which have not been pre-treated with alkali or acid. The glass electrode will be found very convenient when pH values require checking.

[6] 5 p.c. is a mean value. The actual value lies between 4 p.c. and 6 p.c., depending upon temperature and the magnitude (and thus the time) of the titration, because during this time the formaldehyde is still reacting with untitrated ascorbic acid.

the presence of extraneous reducers like quinol, ferrous salt, reductone, etc., curves A_3 and B_3 may be considerably above the baseline.

For general purposes it seems desirable to base calculations upon 10 p.c. persistence of time t_4 (= 4 sec. at 30° C.; 5 sec. at 25° C.; 6 sec. at 20° C.; 7·5 sec. at 15° C.; 9 sec. at 10° C.). The amounts of dye solution needed to give this persistence are read from the titration curves or obtained from the titration data if these have not been graphed, and in each case is added the correction 0·03 ml. (or 0·015 mg.); but if a titration is so small that the "persistence" of the first drop exceeds time t_4 the amount added must be reduced correspondingly (e.g. only 0·01 ml. or 0·005 mg., if the time is $2{\cdot}5 \times t_4$). These corrections are obtained from the titration curves for 0·5 mg. of ascorbic acid, and are sufficiently accurate over the titration range recommended; but strictly speaking the corrections are greater the greater the final volume and are thus dependent upon the magnitude of the titration. Occasionally, particularly in A_3 or A_3'' titrations, it may be clear that the corrected value itself must be less than a single drop, and it may then be determined sufficiently closely by appearance.

It may be pointed out that the influence of a dulled or a supersensitive perception upon the period for the estimated 10 p.c. persistence which is recommended, is not large. Thus, if the period for 10 p.c. persistence is 5 sec., it is about 4 sec. for 20 p.c. persistence and about 6 sec. for 5 p.c. persistence.

Special occasions may warrant the basing of calculations upon 10 p.c. persistences of time less than or greater than t_4. In general, the precision is greater the longer the "persistence", but the scope for systematic error due to extraneous reducers, is also increased.

The corrected titration values may be designated A_1, A_2, A_3, B_1, B_2 and B_3. Then $(A_2 - A_3) \times \frac{100}{93}$, and $(B_2 - B_3) \times \frac{100}{93}$, respectively, represent the amounts of dye needed to titrate the ascorbic and the "total" ascorbic acid contained in the 10 ml. aliquots taken from the flasks A and B, that is to say, in 6·4 ml. of the original extract. If reported as mg. of free dye they are divided by 1·52 (or if reported as ml. of dye solution they are divided by 3·04) to give mg. of ascorbic acid.

By taking 6·4 ml. aliquots of the original extract and diluting each to 10 ml. with water, corrected titrations A_1' and A_2' can be made, corresponding with A_1 and A_2 respectively. They should be identical with A_1 and A_2 but may be greater if adequate precautions to exclude air from the mixture at pH 3·5 have not been taken (particularly if enzyme systems are active or if powerful extraneous reducers are present). If such discrepancy between A_2 and A_2' is known to be due to catalysed aerial oxidation of the ascorbic acid in the case of A_2, then clearly A_2' should be used in place of A_2 in the calculations.

On the other hand, if overwhelmingly large quantities of ferrous salts should be present, advantage may be taken of the great difference in the rates of aerial oxidation of ascorbic acid and ferrous salts in "metaphosphoric" acid solution at pH 3·5. Instead of keeping the solutions at pH 3·5 washed with nitrogen, air is blown through for 1 hr. at about 20° C. and then nitrogen is blown through again before proceeding with the analyses. Under these conditions virtually all the ferrous salt will be oxidized by the air whereas the extent of oxidation of the ascorbic acid will not be detectable unless special catalysts (e.g. oxidizing enzymes, or large amounts of Cu salts) are present. From the titrations (A_1'', A_2'', A_3'', B_1'', B_2'' and B_3'') the ascorbic and dehydroascorbic acid contents are calculated in the usual way.

Obviously much useful, critical information can be obtained by performing all the titrations mentioned (A_1, A_1', A_1'', A_2, A_2', A_2'', A_3, A_3'', B_1, B_1'', B_2,

B_2'', B_3, B_3''), but circumstances will seldom warrant the preparation of sufficient extract for so many titrations.

Titrations A_1, A_2, A_3, B_1, B_2 and B_3 at least, are strongly recommended with materials which have not been submitted formerly to critical analyses.

Simplified Procedures.

Titrations A_1, A_1', A''_1, B_1 and B_1'', are not incorporated in the final calculations and may often be omitted, but it must be appreciated that they provide valuable evidence as to the presence or absence of certain extraneous reducers, and thereby permit a rough estimate of the reliability of the results.

Less critical analyses can be made by ignoring the possibility of the presence of substances like quinol, reductone, and ferrous salts, and omitting titrations A_3 and B_3. In such cases the amounts of dye representing the ascorbic acid and "total" ascorbic acid, respectively, are

$A_2 \times \frac{100}{95}$ and $B_2 \times \frac{100}{95}$.

TESTS AND APPLICATIONS OF METHODS.

In Fig. 3 are the A_1, A_2, A_3, B_1, B_2 and B_3 drop-"persistence" titration curves at 25° C. of various single substances as indicated in the accompanying legend. In many cases the general characteristics of the curves could be inferred from the earlier work (Figs. 1 and 2).

It will be noticed that thiourea (rather unexpectedly) and H_2S-treated 2–Me–1, 4–naphthaquinone belong to the Class 3 substances. Thiourea titrates sluggishly at pH 1·5 and scarcely at all at pH 3·5, and if it were the only extraneous reducer in a test solution of ascorbic acid, its effects might be inappreciable in an A_1 type of titration made at pH 3·5. The reducing substance in H_2S-treated 2–Me–1, 4–naphthaquinone solutions, which titrates sharply and is readily oxidized by exposure to air, appears to be the corresponding naphthaquinol (E_0', + 0·33 volt at pH 1·5, + 0·22 volt at pH 3·5, calculated from the data of Lugg, Macbeth and Winzor, 1936).

It is clear that in order to correct for the interference of Class 3 substances, titrations A_2, A_3 and B_2, B_3 must be used in the calculations. The interference of Class 1 substances is usually reduced to very small dimensions if A_2 and B_2 are used in the calculations and is virtually eliminated if A_2, A_3 and B_2, B_3 are used.

Curves $25A_1$, $25A_2$ and $25A_3$ in Fig. 4 are the A_1, A_2 and A_3 curves respectively for a mixture of 1 mg. $Na_2S_2O_3$, $5H_2O$; 2 mg. $Na_2S_2O_5$; 4 mg. cysteine HCl; and 16 mg. H_2S-treated pyruvic acid. Curves $26A_1$, $26A_2$ and $26A_3$ are for the same mixture with 0·5 mg. ascorbic acid in addition. The titrations were made at 25° C. The following calculations of the ascorbic acid present (or apparently present) may be made from the titration values at the recommended drop-"persistences": $25A_2$, 0·025 mg.; $25A_2$, $25A_3$, 0·005 mg. (actual, 0·000 mg. in each case); $26A_2$, 0·540 mg.; $26A_2$, $26A_3$, 0·515 mg. (actual, 0·500 mg. in each case). Less satisfactory estimates would have resulted from the use of titration values at longer drop-"persistences". If titrations $25A_1$ and $26A_1$ had been used even at the short "persistences" recommended, they would have yielded the utterly misleading values, 0·665 mg. and 1·155 mg. respectively.

The methods have been applied satisfactorily to materials such as cabbage autoclaved with vinegar, dried and germinating cereal and legume seeds, dried plant leaves, human urine and guinea-pig faeces. In the last-named case fairly clear extracts were obtained with the aid of a small centrifuge operating at 20,000 × *g*, and these were rather easier to titrate than were the highly opaque extracts obtained by centrifuging at 2,000 × *g*.

Titrations of the extracts of the treated cabbage (27) and germinating "blue-boiler" peas (28) are represented in Fig. 4. The effects due to extraneous reducers were much smaller than those encountered with the test mixtures (25 and 26). The treated cabbage is estimated to have contained 0·225 mg. ascorbic acid and 0·315 mg. "total" ascorbic acid per gm.; and the germinating peas, 0·27 mg. ascorbic acid and 0·35 mg. "total" ascorbic acid per gm.

Frequent tests have shown that the inclusion of even as much as 20 mg. of SO_2 (29·7 mg. $Na_2S_2O_5$), is without appreciable effect upon the A_2 and A_3 titrations of extracts of fruits and vegetables.

FURTHER CONSIDERATIONS.

In a solution containing ascorbic and dehydroascorbic acid, and fairly well ionized ferrous and ferric salts, there can be only a trace of ferric salt if appreciable quantities of ascorbic acid are present. If "metaphosphoric" acid be added to such a mixture, ferric salt will be formed and there will be very little dehydroascorbic acid if appreciable quantities of ferrous salt remain. It should be recalled that certain organic acids (notably oxalic acid) which occur in some biological tissues, can have much the same effect as "metaphosphoric" acid has upon the ferrous: ferric system. The interpretation of analyses when considerable iron salts are present, therefore, tends to be speculative.

McHenry and Graham (1935), Guha and Pal (1936), Reedman and McHenry (1938) and others have claimed that ascorbic acid occurs partly in a bound or esterified form in natural foodstuffs, and that it can be liberated by heat treatment or by mild acid hydrolysis. Mack and Tressler (1937), Bessey (1938) and others have contended that the experimental evidence should be interpreted differently and that the "bound" ascorbic acid is an artifact.

Interest in claims that ascorbic acid occurs combined in biological material has been revived by Krishnamurthy (1941), who considers that ascorbic acid may occur in combination with formaldehyde in some plant tissues. Then again, Drake, Smyth and King (1942) have shown that dehydroascorbic acid will condense reversibly with sulphhydryl compounds.

It is not yet known whether the condensation products of ascorbic acid with aldehydes (Krishnamurthy, 1941, found no condensation with acetaldehyde or paraformaldehyle) and of dehydroascorbic acid with sulphhydryl compounds, are physiologically useful anti-scorbutic substances. The reversibility of the known condensation reactions suggests that they would be useful, and if this should prove to be the case then any estimation of the anti-scorbutic value of a material should provide for the inclusion of these condensation products.

There are obvious reasons for supposing that in the H_2S treatment of extracts for the reduction of dehydroascorbic acid, the condensation products would yield ascorbic acid. The supposition was checked in the case of the ascorbic acid-formaldehyde condensation product: By allowing 2 mg. ascorbic acid to react with 210 mg. formaldehyde in 100 ml. of solution at pH 3·5 and at 25° C. for about 1 hr., 80 p.c. of the ascorbic acid condensed. When the reaction mixture was treated with H_2S in the usual way for two hours 39 p.c. of the condensed ascorbic acid was liberated. Presumably more would have been liberated if the H_2S had been allowed to react longer, or if the pH of the mixture had been increased. Incidentally, in the work with the extract of treated cabbage (27), some estimations were made with the time, t_3, increased from 2 hr. to 24 hr., but without detectable effect upon any of the titrations.

There is evidence in the literature that as far as the estimation of free ascorbic acid is concerned, t_3 could be reduced to 0·5 hr. or less. Accordingly, by varying t_3 widely, it might be possible to distinguish between the free and combined forms of the substances in an extract.

Finally, it is conceivable that by condensation with — SH groups, some dehydroascorbic acid might be associated with protein in a material, and thus be missed in the extraction with "metaphosphoric" acid. Prior heating in dilute HCl solution, recommended by Reedman and McHenry (1938), might be expected to lead to the breaking down of such combinations, the — SH groups undergoing oxidation by the combined dehydroascorbic acid. Actually there was some loss, both when germinating peas and pure ascorbic acid were so treated.

SUMMARY.

The extraction of ascorbic and dehydroascorbic acids from biological materials is discussed, and methods are described for estimating these substances in the "metaphosphoric" acid extracts (the dehydroascorbic acid after reduction to ascorbic acid by H_2S treatment).

The methods make use of the fact that the reducing substances which may be encountered in the extracts, may be divided into three classes according to their behaviour with formaldehyde. Substances of Class 1 (comprising sulphites, thiosulphates, cysteine, H_2S, H_2S-treated pyruvic acid, etc.) readily "condense" (i.e. form feebly reducing or non-reducing substances with formaldehyde) at pH 3·5 or pH 1·5. Substances of Class 2 (of which the only member so far encountered is ascorbic acid) "condense" readily enough at pH 3·5 but only very slowly at pH 1·5. Substances of Class 3 (comprising quinol, H_2S-treated 2–Me–1, 4–naphthaquinone, thiourea, reductone, ferrous salts, etc.) do not "condense" appreciably at either pH 3·5 or pH 1·5.

The "condensations" are carried out under carefully controlled conditions. The reducing capacities of the reaction mixtures are then measured under standard conditions: preferably by titration at pH 1·5 with a standard solution of 2, 6–dichlorphenolindophenol in drops of definite size to a point at which the colouration due to a drop persists for a standard time down to a predetermined fraction of its initial intensity. From these "drop-'persistence' titrations" the ascorbic acid and dehydroascorbic acid contents are easily calculated.

The full method is believed to be extraordinarily specific, ascorbic acid being the only substance found yet in Class 2.

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ANTIBACTERIAL SUBSTANCES PRODUCED BY MOULDS

1. PENICIDIN, A PRODUCT OF THE GROWTH OF A PENICILLIUM.

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(Accepted for publication 3rd November, 1942.)

During an investigation of the production of antibacterial substances by moulds (Atkinson, 1942) a penicillium (Mould 9) was found which produced a substance inhibitory to both Gram-positive and Gram-negative bacteria. The present paper describes the method of production and extraction, and some of the properties of this substance for which the name "penicidin" is used throughout.

Method of Production.

The medium used was that of Czapek-Dox modified as described by Abraham *et al.* (1941) for the production of penicillin, and was distributed in bottles in a shallow layer not more than 2 cm. deep. It was inoculated with a suspension of spores and incubated at 18–20° C. The course of production of penicidin was followed daily by a modification of the method of assay described by Abraham *et al.* (1941) for penicillin. The peak of production was reached about five days after inoculation and the fluid was then removed and replaced with fresh medium using aseptic precautions. The production of penicidin in the replaced medium was more rapid than in the primary culture, being at its peak three to four days after replacement. It was possible to utilize the same mycelial felt for several such replacements; the replaced fluid was harvested on the third day after replacing. If the primary or replaced cultures were incubated for a longer time than the optimal indicated above, the penicidin gradually disappeared. After removal from the mould, the metabolism solution (Raistrick's terminology) was filtered through a Seitz bacterial filter and stored at 3° C. until required.

Extraction of Penicidin.

The metabolism solution was concentrated *in vacuo* to about one-tenth of its original volume, filtered, adjusted to approximately pH 7 and extracted continuously with ether for several hours; by this time all the penicidin had been taken up by the ether. The ether extract which was a very pale brown was then washed with three or four volumes of water adjusted to pH 4 by addition of H_2SO_4. The penicidin was removed from the ether-extract by the dilute acid, giving a faintly yellow solution which on neutralization showed a characteristic change to a pale bronze colour. However the substance responsible for this colour was not penicidin, as extraction of the neutral bronze solution with ether resulted in the removal of all the penicidin while the colour of the watery solution remained unchanged and no colour appeared in the ether. The ether extract was thoroughly dried, evaporated *in vacuo* to small volume and three or four volumes of petroleum ether added. There was an immediate cloudiness which on standing in the cold almost completely settled out as a pale yellow oily layer on the bottom of the beaker. This oily material was the penicidin, probably incompletely purified. It inhibited the growth of *Bact. typhosum* in a dilution of approximately 1/100,000.

Some Properties of Penicidin.

(a) *Heat-resistance.* In metabolism solution adjusted to pH 7, a small amount of destruction took place after steaming for 30 minutes and further destruction occurred after steaming for three hours. However, some activity still remained even after this treatment. No destruction occurred during evaporation of the metabolism solution *in vacuo* at a temperature not exceeding 45° C., nor was there any destruction of the penicidin after boiling in ether for two hours. Penicidin is therefore relatively heat-resistant in neutral solution.

(b) *Resistance to HCl and H_2SO_4.* Penicidin was soluble in dilute HCl or H_2SO_4, pH 4, without obvious loss of activity. No obvious destruction could be detected after 40 minutes in metabolism solution which had been strongly acidified (pH 3) with either HCl or H_2SO_4. Penicidin was therefore not readily destroyed by these acids.

(c) *Resistance to KOH and $Ba(OH)_2$.* In metabolism solution adjusted to pH 7·8 with KOH no alteration in activity took place after one hour. However, when the fluid was made strongly alkaline with KOH the activity was completely destroyed in a few minutes. Also treat-

ment of a crude active ether extract with $Ba(OH)_2$ resulted in the formation of a precipitate and the complete loss of activity which could not be demonstrated in either the precipitate or the liquid. Thus penicidin is fairly easily destroyed by these bases.

(d) *Solubility.* Penicidin is soluble in chloroform, benzene, ether, ethyl alcohol and dilute mineral acids. It is not soluble in petroleum ether.

(e) *Results of some qualitative chemical tests.* A solution of the purified penicidin in 5 p.c. ethyl alcohol gave a negative reaction to the biuret, Molisch and Millon's tests.

The addition of a small amount of 10 p.c. NaOH to the solution produced a very pale yellow colour which gave a yellow fluorescence in ultraviolet light.

No reaction took place on addition of a small quantity of $FeCl_3$. With slight excess of this reagent a faint purplish brown appeared but disappeared again with gross excess. No further change took place on heating.

(f) *Other properties.* From metabolism fluid penicidin readily dialysed through cellophane into distilled water at 3° C.

Penicidin is readily adsorbed from metabolism fluid by activated charcoal.

DISCUSSION.

It is unlikely that the penicidin prepared as described is completely purified. In the early extraction procedures it is accompanied by at least two other substances, both giving coloured solutions, and the yellow colour and fluorescence in alkaline solution of the purified penicidin may be due to contamination with a small amount of these pigments. However, solid derivatives are being prepared and should assist in elucidating the chemical nature of penicidin which, so far, has not shown itself to be a very reactive substance.

SUMMARY.

The method of production and preparation and some of the properties of an antibacterial substance, called here penicidin, have been described.

Acknowledgment. I am greatly indebted to Mr. E. L. French for his valuable assistance in the performance of this work.

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THE LIBERATION OF DEAMINATING ENZYME IN PNEUMONIA

by E. R. TRETHEWIE[1]

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(Accepted for publication 11th October, 1942.)

It has been shown by Drury, Lutwak-Mann and Solandt (1938) that human plasma contains an agent capable of inactivating adenyl compounds. The toxins of *Cl. welchii* (Kellaway, Trethewie and Turner, 1940; Kellaway and Trethewie, 1941) cause the liberation of a similar agent from the isolated perfused liver, and in injury by lower concentrations of chloroform inactivating enzyme is more readily detected than are adenyl compounds or histamine (Trethewie, 1941). It was therefore thought worthwhile to determine if an increase of inactivating enzyme in the blood occurred in pneumococcal infection.

METHODS.

The estimation of inactivating enzyme in plasma. Using a dry 10 c.c. syringe blood was withdrawn from the median basilic vein in human subjects immediately after applying a tourniquet to the arm. The blood was added to a dry test tube containing heparin (0·12 cc. or 600 units to 7 c.c. blood). The supernatant plasma separated by centrifugation was pipetted off into a sterile test tube. Lacarnol, a standardized preparation (Bayer) containing adenyl compounds, was added to the plasma to make a solution of 1 part in 50 by volume. To maintain accuracy, the lacarnol was first diluted 1 in 5 with Tyrode solution. The activity of the plasma was measured by estimating the percentage of lacarnol remaining active after 20 and 40 minutes incubation at 39° C. (maintained within 0·5° C.). For this purpose the atropinized guinea-pig heart preparation of Drury, Lutwak-Mann and Solandt (1938) was used.

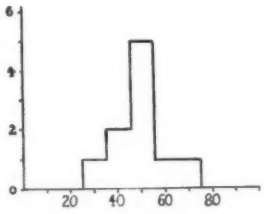

Fig. 1. Histogram of distribution of enzyme activity in normal plasma. Ordinates: number of cases; abscissae: p.c. inactivation lacarnol after 40 minutes incubation.

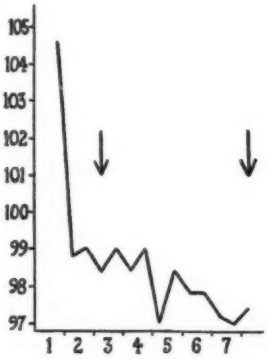

Fig. 2. Temperature chart of a case of pneumonia. At the arrows samples of blood taken for testing. Details in text.

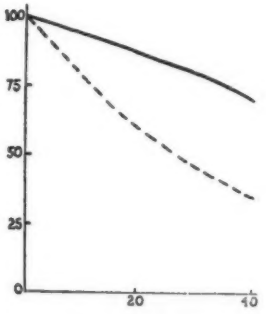

Fig. 3. Activity of plasma from case of Fig. 2. Ordinates: p.c. lacarnol remaining active; abscissae: time of incubation in minutes (39° C.). Continuous line: sample taken at second arrow Fig. 2. Interrupted line: sample taken at first arrow.

[1] This work was aided by a grant from the National Health and Medical Research Council.

The pH of the plasma was measured before and after incubation by a glass electrode using 0·4 c.c. volumes. Measurement was accurate to within 0·03 pH. It was maintained at a nearly constant level by passing over the system a continuous stream of 4·6 p.c. CO_2 in oxygen saturated with water vapour. If this is not done the pH rises rapidly. During incubation the variation was usually less than 0·08 pH. The average of these figures was taken as the pH of the system. Different plasmas were incubated at slightly different pH, the variation being 7·5–7·9, but repeat tests of any one plasma were within 0·1 pH of the earlier test. Except in one instance the blood was obtained at the same time each day.

RESULTS.

The Content of Inactivating Enzyme in Normal Human Plasma.

It is necessary firstly to determine the amount of inactivating enzyme in plasma from healthy subjects and secondly the variation with time. The subjects chosen were fracture cases who had been in hospital for some weeks and were not undergoing active treatment and also healthy patients admitted for hernial operation. The extreme variation in eight of the former and two of the latter was 30 p.c.–70 p.c. (see method) with an average 50 p.c. activity remaining after 40 minutes incubation. A histogram of the distribution for 10 p.c. differences in activity is shown in Fig. 1. Five of the ten cases yielded plasma with a 50 p.c. activity level.

When estimations were made on two occasions at intervals of 2 to 13 days no obvious changes were observed in the activity of plasma from fracture patients lying in bed in splints weeks after the injury, or from other healthy patients (Table 1, Control observations). The last three control figures in the table were obtained in a single patient.

TABLE 1.

Percentage of lacarnol remaining in system after 40 minutes incubation.

Pneumonia.			Control observations.		
Before Recovery.	After.	Interval between testing.	First.	Second.	Interval.
17·5	25	4 days	48	45	2 days
			60	50	2 ,,
			53	58	3 ,,
32·5	70	5 ,,	40 *	34	6 ,,
27	48	7 ,,	34	36	7 ,,
27	55	13 ,,	40	36 *	13 ,,
26%	50%	Average	50%	50%	Average

Samples * . . . * were all obtained from the same patient.

The variation with pH change. The variation in pH in the experiments recorded did not significantly affect the activity of the enzyme. In an experiment in which the same plasma was tested under different conditions of pH (by excluding CO_2 from one system) a rise of pH of 0·36 gave an increase in activity after 40 minutes from 48 p.c. to 40 p.c. In another experiment in which the pH change was 0·26 the activity varied from 50 p.c. to 45 p.c. In the recorded experiments the variation did not usually exceed 0·08 pH.

The Content of Inactivating Enzyme in Patients Suffering from Pneumonia.

Immediately following the febrile stage in pneumonia the enzyme content of the plasma is high, but as the patient regains his feeling of well-being the content returns to an average normal figure. At the height of the acute illness it has not been thought advisable to subject the patient to bleeding. Blood was obtained as soon as the temperature began to fall when the patient was still obviously ill. In the four cases investigated the activity remaining in the plasma (lacarnol) after 40 minutes incubation was 17·5, 32·5, 27 and 27 p.c. These figures give an average of 26 p.c. which is significantly lower than the normal (50 p.c.). When the patients became afebrile and looked well, estimations made 4, 5, 7 and 13 days later were 25, 70, 48 and 55 p.c. respectively. The low figure (25 p.c.) was obtained from a boy aged 14 years; the remainder were adults. No control observations have as yet been made in children. These changes when compared to the relative constancy of the control cases (Table 1) are significant. The detailed findings for the second patient in Table 1 were as follows. He was suffering from pneumonia (pneumococcus type 1) affecting the left base and his general symptoms were severe with a temperature of 104·6° F. Radiography confirmed the diagnosis. Two days later the temperature had fallen to 99° F. (see Fig. 2) at which stage plasma was obtained. The plasma content of lacarnol fell to 60 p.c. after 20 minutes and to 35 p.c. after 40 minutes incubation (interrupted line, Fig. 3). The patient still felt sick. Five days later, the temperature having remained normal (see chart) the activity of the plasma was much less, the figures being 87 p.c.

and 70 p.c. respectively (continuous line, Fig. 3). However, five days later he suffered a relapse due to an extension of the pneumonia and again felt and looked ill. At this stage the enzyme content was increased, the figures for the plasma content on incubation being 62 p.c. and 42 p.c. respectively. If we regard the value for the interim "well" period as the normal plasma content for this patient, then the increased activity at the initial testing soon after the onset of the pneumonia can be expressed as $\frac{70-35}{70} \times 100$ or an increase of 50 p.c. and at the recrudescence an increase of 40 p.c.

Since the sulphonamide drug M and B 693 was used therapeutically in all these cases it was necessary to exclude the possibility that the change in activity of the plasma might have been related to the presence of this compound. This was tested as follows. Plasma was divided into two portions and M and B 693 previously mixed with Tyrode 2 mg. per c.c., was added to one in the concentration of 7·5 mg. per 100 c.c. plasma, the maximum amount present in these patients. Incubation with lacarnol was then continued with both. The activity of the plasma at the end of 40 minutes incubation was 35 p.c. without and 40 p.c. with M and B 693, i.e. there was a slight diminution in enzyme activity, the reverse effect to that produced in pneumonia. In two other experiments in which M and B 693 was incubated with the plasma in concentrations of 7 mg. and 8 mg. per 100 c.c. the figures for one were 60 p.c. without and 62 p.c. with sulphanilamide, and in the other 65 p.c. and 58 p.c. respectively.

The Presence in the Plasma of Another Cardio-depressant Substance.

In the plasma from three of the four patients suffering from pneumonia there was a cardiac depression which followed the response to the added lacarnol and which was longer in duration but not so deep. A similar effect is seen when lacarnol is given to a struggling heart which has a poor excursion, though with recovery of the beat this disappears; or may be produced on the healthy heart by the injection of particulate matter into the auricle. The effect here observed is not attributable to adenyl compound for it is not destroyed by the inactivating enzyme. This is shown in Fig. 4. At 2 is shown the response of the plasma, with added lacarnol, before incubation and at 1 it is seen that the immediate depression has almost completely disappeared following incubation but the secondary effect is unchanged. It cannot be attributed to sediment because the boiled mixture was allowed to settle in a tall tube before injection. In the three patients suffering from pneumonia whose plasma reacted in this way, in two the effect had disappeared at the time of the last testing (10–13 days later) but in the third it was still present when the second test was made after four days.

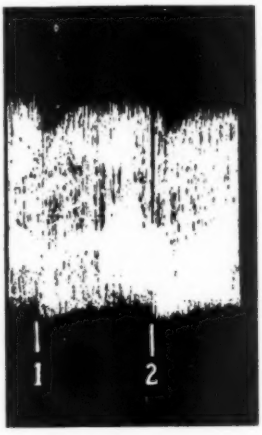

Fig. 4. Response of atropinized guinea-pig auricle to sample containing cardio-depressant substance other than adenyl compound. Details in text.

This effect was observed in only one of the eight long-standing fracture cases among the controls. It was present twelve weeks after the accident, but two weeks later had disappeared. It was not observed in either of the hernia cases.

DISCUSSION.

In investigations designed to determine the production of symptoms by infective and toxic processes in addition to reflex nervous effects, two modes of action have been recognized. In one the injurious agent is believed to act directly, e.g. the coronary constrictor property of the *Cl. welchii* group of toxins, while in the other numerous indirect effects are thought to be determined by the liberation of pharmacologically active substances most of which are already present preformed in the tissues.

That this latter mode of action is important in anaphylaxis has been well demonstrated by Rosenthal and Brown (1940). The liberation of histamine in this condition was demonstrated by Bartosch, Feldberg and Nagel (1932) and also by Dragstedt and Gebauerfuelnegg (1932), and as was suggested earlier by Dale and Laidlaw (1910) the fatal bronchoconstriction in the guinea-pig can be explained by the liberation of histamine. Rosenthal and Brown (1940) were able to show that if thymoxethyldiethylamine be injected prior to the exhibition of the

anaphylactic antigen the pulmonary effects were practically abolished and the outcome was no longer fatal. This substance has been shown to inhibit the pharmacological actions of histamine both in the intact animal and on isolated organs. It is reasonable to consider that further substances may be discovered which will inhibit the actions of other pharmacologically active substances released in various conditions, and therefore it appears desirable to determine the production of the symptomatology more fully. It is not to be expected that all of the substances so liberated are equally important as regards the severity of the symptoms; e.g. in anaphylaxis it has been shown (Kellaway and Trethewie, 1940) that in addition to histamine a slow reacting muscle stimulant substance is liberated though toxic properties have not as yet been ascribed to this substance.

In injury by limiting concentrations of chloroform, the only active substance readily detectable in the perfusate from isolated organs is a deaminating enzyme. Possibly this substance may account for some of the symptoms. In pneumonia the similar increase in this activity in the blood may contribute to the production of symptoms. There is at least a correlation between the malaise of the patient and the content of this enzyme in the blood. On the other hand the increase in enzyme activity may be due to the presence of other substances, liberated in injury, which activate the enzyme.

When specific soluble substance of type 1 pneumococcus prepared by the method of Dudley and Wilson Smith (1933) is injected intraportally into the isolated perfused liver of the rabbit, adenylic acid and inactivating enzyme are liberated into the hepatic vein (Trethewie, 1942). It is therefore possible that some of the increased enzyme content appearing in the blood of the patients is derived from the liver. The capsular extract used in these experiments was supplied by Dr. Wheatland of the Commonwealth Serum Laboratories, Melbourne.

The second cardio-depressant substance detected in the plasma of patients suffering from pneumonia has not been further investigated. This effect is not due to adenyl compound because it is not inactivated by deaminating enzyme. It may be related to the circulatory effect of serum described by Brodie (1900), which in the case of *Cl. welchii* antitoxin can be removed by precipitation of the globulin fraction and dialysis (Kellaway and Trethewie, 1941). Bick and Reid (1942) have shown that the noxious effects of serum are due to two substances, one dialysable and the other non-diffusible. They have also shown that platelet extract can reproduct this effect, and serum obtained without platelets is free of this activity. Since there is a platelet-reducing fraction in pneumococci, and purpura occurs in some patients suffering from pneumonia, it is possible that this cardio-depressant substance is of platelet origin.

SUMMARY.

The activity of deaminating enzyme in plasma from normal human subjects has been assessed.

The variation in activity of this plasma over short periods and for slight changes in pH has been determined.

An increase in enzyme activity has been found in the blood taken from patients suffering from pneumonia.

A cardio-depressant substance other than adenyl compound has also been detected.

The significance of these findings is discussed.

Acknowledgment. Thanks are due to the Honorary Staff of the Royal Melbourne Hospital for access to patients.

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STUDIES IN THE EPIDEMIOLOGY OF Q FEVER

11. EXPERIMENTAL INFECTION OF THE TICKS *HAEMAPHYSALIS BISPINOSA* AND *ORNITHODORUS SP.* WITH *RICKETTSIA BURNETI*[1]

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(Accepted for publication, 20th October, 1942.)

TRANSMISSION OF Q FEVER WITH *H. BISPINOSA*.

Haemaphysalis bispinosa Neumann (1897) infests cattle in the coastal districts of southern Queensland and northern New South Wales. Engorged female ticks obtained from butcher's sheep at Buderim, South Queensland, and from cows at Kempsey, New South Wales, were found to be free from infection with Q fever. They and their progeny were used in the transmission experiments summarized below.

It was found that *H. bispinosa* could become infected with Q fever by feeding on infected animals in its larval, nymphal or adult female stages, and that infection passed from larva to nymph and from nymph to adult.

Fifty-three nymphs, previously fed as larvae on an infected rabbit, produced infection in a rabbit upon which they fed.

Other nymphs, which had been infected as larvae on guinea-pigs, were fed in batches of from one to ten on eight guinea-pigs. One guinea-pig, on which seven nymphs engorged, became infected.

Forty-four adult females, exposed to infection as larvae or nymphs, were placed in capsules on eight guinea-pigs. Only fifteen made any attempt to feed and none engorged fully. None of the host animals became infected.

In infected female ticks examined by serial sections, rickettsiae were often abundant. They appeared to be limited to the lumen and lining epithelium of the midgut and its caeca. In infected cells rickettsiae were seen only in the cytoplasm; invasion of the nuclei was not observed.

One hundred and five adult ticks developed from ova laid by the ticks captured at Buderim and Kempsey. All were females and died or were killed before ovipositing. It was not possible, therefore, to test for hereditary transmission of *Rickettsia burneti*. Myers (1924) has commented on the extreme rarity of males of *H. bispinosa*.

DISCUSSION.

As *H. bispinosa* is a three-host tick occurring on cattle in the Q fever area (though much less frequently than *Boophilus annulatus microplus*), and as cattle in this area suffer naturally from Q fever (Derrick, *et al.*, 1942), it seems likely that *H. bispinosa* plays a part in spreading infection from cow to cow. It is possible also that *H. bispinosa* carries infection from bush animals to cattle. Although we have not found this tick on bush animals, in other countries it has a wide range of hosts.

THE PERSISTENCE OF *R. BURNETI* IN *ORNITHODORUS SP.*

In October, 1940, seven ticks (one adult and six nymphs, provisionally identified as *Ornithodorus gurneyi*), collected in a cave at Mt. Isa, N.W. Queensland,

[1] This paper, although incomplete, is presented for publication since investigations into the entomological aspects of Q fever have now been suspended.

were fed upon febrile guinea-pigs infected with Q fever. Subsequently some of them were fed upon normal guinea-pigs at intervals ranging from 20 to 143 days, and inoculated individually into 6 guinea-pigs at intervals ranging from 129 to 538 days.

No guinea-pig upon which the ticks fed became infected. This result is probably correlated with the unlikelihood of the wound being contaminated with infected material from the hind-gut during the short time spent on the host; the ticks became fully engorged within 25 to 75 minutes. The coxal fluid which is often expelled before detachment is apparently not infective.

Individual ticks inoculated 129, 135 and 538 days after their infective feed infected the recipient animals. The virus appeared to be of undiminished virulence.

Acknowledgments. I am indebted in various ways to Sir Raphael Cilento, Dr. E. H. Derrick, Professor H. R. Seddon, Dr. F. H. S. Roberts, Mr. Max Henry and Mr. A. E. Moore.

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EXPERIMENTAL DEMYELINATION OF THE CENTRAL NERVOUS SYSTEM

3. POISONING WITH POTASSIUM CYANIDE, SODIUM AZIDE, HYDROXYLAMINE, NARCOTICS, CARBON MONOXIDE, ETC., WITH SOME CONSIDERATION OF BILATERAL NECROSIS OCCURRING IN THE BASAL NUCLEI[1]

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(Accepted for publication, 24th August, 1942.)

An earlier paper (Hurst, 1940), frequently referred to below, described the condition produced in the brains of monkeys given repeated large doses of KCN over periods varying from 3 weeks to about 3 months. Under the conditions obtaining in these experiments, necrosis of the white matter occurred more frequently than did the simple demyelination described by Ferraro (1933a and b). In a remarkable manner, it often occurred suddenly and simultaneously over considerable areas of the cerebral hemispheres, and in distribution copied closely the lesions of Schilder's encephalitis in men and monkeys. This necrosis rarely followed single doses of KCN; these more frequently caused extensive damage to the grey matter of the cerebral cortex, basal ganglia or cerebellum.

Amongst other things the present paper deals with the effects of KCN administered in rather smaller doses over longer periods, as well as with those of other chemicals used by biochemists to inhibit intracellular oxidation-systems. In the course of the work, light has possibly been thrown upon the symmetrical necrosis of the basal ganglia reported in a variety of intoxications in man.

FURTHER OBSERVATIONS WITH POTASSIUM CYANIDE.

(i) *Preparation of solutions for injection.* When powdered, the balls of KCN supplied commercially emit a strong odour of HCN. In earlier attempts to avoid irregularities due to the presence of variable amounts of the acid, we powdered the balls and kept the powder in Erlenmeyer flasks in the cold-room, in quantities of a few hundred mg., for not less than a week before use. In the present experiments we dissolved at one time a large quantity of KCN and stored the solution in sealed 10 c.c. ampoules. Probably because of its greater HCN content, the lethal dose of this solution was lower than that of the solution prepared previously. Irregularities of reaction on the part of the recipients were not wholly eliminated. On Mondays particularly, following a free Sunday, monkeys tended to suffer much longer periods of unconsciousness than after the same dose of KCN administered on the days preceding the week-end.

(ii) *Intoxications over relatively long periods.* Twelve monkeys (*M. mulatta*) on an adequate diet received intramuscular injections of KCN on six days each week. They were weighed weekly. The initial dose of 3 mg. per kilo. was raised to cause daily periods of unconsciousness each averaging about 45 minutes. Three monkeys died (on Mondays) after 59, 79 and 82 days, respectively. The remainder survived for from 105 to 185 days; they were killed when obviously sick after receiving in all from 400 to 760 mg. KCN per kilo. body-weight.[3]

In these animals the lesions differed substantially from those recorded previously, in that they resembled more closely those described by Ferraro.

The basal ganglia, cerebral cortex, cerebral white matter and optic tracts showed no important necroses, though occasionally the basal ganglia were the seat of lesions characterized by pure demyelination. The cerebellum seldom exhibited the changes previously described as occur-

[1] A preliminary account of part of this work was published in the First Rennie Memorial Lecture (Hurst, 1941).

[2] Working with an expenses grant from the National Health and Medical Research Council.

[3] In the earlier article the total amounts of KCN administered were given together with the approximate weights of the monkeys.

ring in the molecular zone and Purkinje-cell layer. On the other hand, the white matter of the cerebrum stained very feebly by Weil's method (Fig. 1). This demyelination extended usually from the frontal to the occipital region, and involved the centrum semi-ovale, the corpus callosum, the anterior commissure, and to a lesser degree the optic tracts. The optic chiasma and optic nerves suffered less than did the tracts; the central fibres of the nerves were most affected. As in Schilder's disease in man, the subcortical fibres were less affected than were those more deeply placed. The internal capsules and optic radiations were comparatively normal. In the most severely affected areas, demyelination was almost complete. In contradistinction to the findings of Ferraro, the white matter of the cerebellum, brain-stem and spinal cord stained normally.

The cellular elements present in the demyelinated areas resembled those in the simian variant of Schilder's disease, though in the two cases of the latter available for comparison (see Hurst and McLennan, 1937) the lesions were focal and of much more restricted distribution than is ordinarily the case in poisoning with KCN. Large fibre-forming glial cells were numerous; associated with them was a moderate number of polymorphic microglial cells and but few granular corpuscles. In preparations stained with scharlach R, the amount of visible fat often seemed disproportionately small when compared with the degree of demyelination. In Weil preparations, not only was there macroscopic pallor of the affected areas, but the surviving myelin sheaths stained more palely than did those in areas such as the pyramidal tracts; we believe that this may have been due partly to over-differentiation in areas where only a few sheaths persisted. Though considerable, the immunity to damage of the pyramidal tracts and optic radiations was only relative; microscopically, numerous sheaths were seen to be ballooned or fragmented. Usually the loss of axis-cylinders was roughly proportionate to that of the myelin sheaths. Some vessels showed slight mononuclear perivascular cuffing; in most areas there was no proliferation or thickening of vessels. In relatively less affected parts of the white matter, but seldom in those most severely demyelinated, numerous "vacuoles" were present; these are described more fully below in the description of the lesions due to sodium azide. Their relative absence from more fully demyelinated areas may possibly be associated with the progressive disappearance of oligodendroglial cells from the regions most affected. It is to be noted, however, that while the oligodendroglia degenerated in affected areas, there was no evidence that its disappearance was the cause and not merely a concomitant of demyelination.

(iii) *Failure of the lesions to progress in the absence of further administration of KCN.* Two monkeys receiving 500 and 700 mg. per kilo. in the course of 150 days, and showing residual nervous symptoms after recovery from the immediate effects of the doses of KCN, were killed 3 and 6 weeks respectively after injections were discontinued. In the meantime the clinical condition of the animals had slowly improved. In the former, ballooned and fragmented myelin sheaths were still evident. In the latter, the appearances were those to be expected from previous intoxication at a fairly remote date. In neither was there any suggestion that the encephalopathy was progressive once injections of KCN ceased.

(iv). *Effect of vaccinial infection on the course of KCN poisoning.* It is well-known that in man an encephalomyelitis characterized by perivascular demyelination is an occasional sequel of vaccinia and other acute infections. The cause is obscure, and comparable lesions have never been reproduced in animals. We were unable to show that poisoning with KCN predisposes to this complication of vaccinia.

Four monkeys in an advanced stage of poisoning with KCN received intradermal injections of vaccinia virus passaged through the testes of rabbits. The injections of KCN were continued during (and after) the vaccinial reaction. Since, however, at the height of this reaction the periods of unconsciousness due to a constant daily dose of KCN increased considerably, in three monkeys a rather smaller dose seemed temporarily necessary to ensure survival. In none, however, were unusual symptoms apparent. When the animals were killed several weeks after the vaccinial reaction, the lesions present in the brain differed in no way from those already described.

(v) *Effects of repeated massive doses of KCN followed by restorative measures.* We noted previously that monkeys dying after single large doses of KCN rarely showed necrosis in the white matter; the principal effect of the poison was on various areas of grey matter. Subsequent observations have furnished two examples of diffuse pallor of part of the white matter (corpus callosum and occipital white matter) due to a single large dose.

Two monkeys received doses of KCN up to twice the lethal; when respiration had virtually ceased, the animals were revived by intravenous injection of sodium nitrite followed by sodium thiosulphate, and if necessary by artificial respiration and injection of adrenalin. They thus received 8 and 10 doses respectively in the course of from 20 to 26 days. After the last doses attempts at resuscitation failed.

On histological examination of the brain, almost every major cortical area proved to be affected; the occipital cortex was particularly severely involved. Numerous areas of partial or complete necrosis, of different ages, lay usually in the outer but sometimes in the middle laminae.

In the former situation the lesions were usually more or less focal, in the latter band-like over considerable distances. Occasionally the whole depth of the cortex was necrotic. The caudate nucleus, putamen, optic thalamus and corpus subthalamicum showed a few small necroses. The cerebellum was severely affected in the manner previously described. The white matter appeared normal.

A further experiment originated in the thought that possibly after repeated large doses of KCN the monkeys became sensitized to altered proteins resulting from the breakdown of their own nervous tissue, and that the response to such sensitivity might be the massive and sudden necrosis of large tracts of white matter. Four monkeys each received on three successive days large doses of KCN; they were resuscitated as before. A month later they were tested with a moderately large dose of KCN. No unusual reaction followed.

(vi) *Staining of the brain with methylene blue in poisoning with KCN.* Following ineffectual attempts to improve, by treatment with methylene blue, the condition of monkeys dangerously ill, we noted that some brains were coloured as long as 4 days after intravenous injection of small amounts of the dye. The doses of methylene blue ranged from 1·5 to 3 c.c. per kilo. of a 1 p.c. solution [or double this amount of a 0·5 p.c. solution in 1·8 p.c. sodium sulphate (Hanzlick and Richardson, 1934)]; they were usually 2 [or 4] c.c. per kilo. This dosage (see Cooke, Hurst and Swan, 1942) is about 3–4 times that recommended for therapeusis in man (Edmunds and Gunn, 1936), and 4–7 times that which Friedemann (1937) found necessary to colour the brains of guinea-pigs of 300 gm. weight within 3 minutes of inoculation. In these experiments the dye was administered about 6 hours after a large dose of KCN and the animal killed after a further 24–96 hours. In 4 such monkeys the colour of the brain on opening the dura was grey or definitely blue, in one case patchily blue. On exposure to air or immersion in formol-saline the grey matter turned more or less uniformly blue; the white matter was more faintly coloured. Two controls killed 24 hours after similar doses of methylene blue showed no colouration either immediately or after exposure to air or to the fixative. In severely poisoned animals, therefore, there was evidence of greater permeability of the blood-brain barrier, or of longer staining through delayed excretion of the dye. Dye penetrating the brain was reduced incompletely to its leuco-base. All these monkeys were continuously very sick from the time of injection of KCN; when killed they were moribund.

These observations together with some others noted previously, such as the unexplained recurrence of nervous symptoms in monkeys several days after recovery from the effects of a dose of KCN, led us to investigate whether damage to the blood-brain barrier is a factor in producing the encephalopathy due to KCN. It may be recalled that in the case of other poisons dealt with below [e.g. CO (Siengalewicz, 1924) and urethane (Landis, 1927)] damage of the blood vessels is recorded. The following observations were on rabbits of about 1,000 gm.

(1) Three normal rabbits showed no colouration of the brain 24 hours after intravenous injection of 2–3 c.c. per kilo of a 1 p.c. solution of methylene blue in 1·8 p.c. sodium sulphate (i.e. of a dose comparable with that used in the experiments on monkeys above). Two hours after a similar dose, the brains of 6 animals were definitely coloured, with slight differences in tint in the individual animals.

(2) Eight rabbits treated similarly 6 hours after 5–8 mg. KCN per kilo. showed no staining of the brain 24 hours later. In one such rabbit the brain was coloured; in contradistinction to the other animals, this had never recovered consciousness after its dose of KCN.

(3) Four rabbits treated similarly 15 minutes after 5–8 mg. KCN per kilo., i.e. during the period of unconsciousness, showed uncoloured brains 24 hours later. The brains of 7 rabbits treated similarly but killed 2 hours after methylene blue were on the whole rather less deeply coloured than were those of the 6 controls mentioned above.

(4) Four rabbits treated similarly 15 minutes after the last of five daily doses of 5–7 mg. KCN per kilo. exhibited unstained brains 24 hours later.

In rabbits, therefore, poisoning with KCN does not demonstrably increase the permeability of the blood-brain barrier, or retention of methylene blue in the nervous tissues, unless the dose is such as to bring the animal to the point of death. In collateral experiments for another purpose, we found that the brain remains coloured with methylene blue when the animal is moribund from other causes (see Cooke, Hurst and Swan. 1942). Furthermore, the brain of a monkey injected with comparable doses of methylene blue during general anaesthesia with A.C.E. was coloured. In severely poisoned monkeys, dye penetrating the brain apparently did not improve the animals' condition; as far as it goes this observation accords with the views of Wendel (1935) on the therapeutic effect of methylene blue, rather than with those of Brooks (1935). It may be mentioned that prior treatment for several days with methylene blue did not raise appreciably the tolerance of monkeys to a dose of KCN amounting to 6 mg. per kilo.; at autopsy the brains were not blue.

(vii). *Poisoning with KCN in the sheep and in the rabbit.* Owing to failure of supplies consequent upon the war, we used sheep instead of monkeys for some of our experiments (see also later). Both sheep and rabbits exhibited much greater

individual variations than did monkeys in response to a given dose of KCN, as well as greater daily variations in response to a constant dose.

Seven sheep received from 115 to 406 mg. per kilo. KCN in the course of from 36 to 110 days. Two died on Mondays, following a week-end free from injections, without increase of the amount previously given. Two showed areas of "partial" damage in the cerebral cortex or basal nuclei, or in both. Two (115 mg. per kilo. in 36 days and 406 mg. per kilo. in 110 days) showed small areas of necrosis of some duration in the parietal or occipital white matter. One (373 mg. per kilo. in 110 days) showed a small area of demyelination in the white matter of the occipital lobe. These lesions in the white matter occurred in much closer relation to the ventricular system than did those in monkeys (cf. Ferraro's results in cats). Two animals (248 mg. per kilo. in 78 days and 373 mg. per kilo. in 110 days) showed beading of the axons and ballooning of the myelin sheaths in a few fibres of the optic nerves and tracts. We received the impression that, for the present purpose, the sheep is a less suitable animal than the monkey.

Comment.

Occasionally (Hurst, 1940) necrosis of the white matter of the cerebrum may follow a single large dose of KCN. Nearly always, however, single and repeated large doses cause major involvement of the cerebral and cerebellar cortex, with less severe involvement of the basal nuclei; there is no constancy in the exact site of cortical lesions either with regard to the chief subdivisions of the hemisphere or to the various cortical laminae. Repeated, rather smaller, doses often produce severe bilateral lesions in the basal ganglia and areas of necrosis in various parts of the white matter, particularly in that of the occipital lobes; in the course of the intoxication, massive necrosis affecting almost all the white matter of the cerebrum may develop suddenly and simultaneously over a very wide area. With repeated still smaller doses over a longer period, the grey matter usually escapes damage. Instead there develop gradually in the white matter lesions, at first characterized by "overcellularity" and an increased number of Marchi-staining droplets, and then by progressive demyelination and loss of axis-cylinders. Demyelination becomes almost complete over most of the centrum semi-ovale, corpus callosum, and anterior commissure. The optic tracts suffer to a lesser degree and the optic nerves still less. To both necrosis and gradual demyelination, the internal capsules and optic radiations are relatively resistant. Both processes are more marked centrally and tend to spare the subcortical U-fibres, though these are less resistant than are those of the internal capsules and optic radiations.

These lesions are not progressive in the absence of further administration of KCN[4]. Their presence does not favour the development of post-vaccinal encephalitis. There is no evidence that damage to the blood-brain barrier plays any part in their genesis.

THE EFFECT ON THE NERVOUS SYSTEM OF OTHER INHIBITORS OF ENZYME-REACTIONS.

In seeking further evidence that demyelination might arise from poisoning with chemical substances of fairly simple pharmacological action, we reviewed the many compounds employed by the biochemist to inhibit one or other enzyme-system *in vitro*. Many difficulties beset an amateurish attempt to make use of these compounds *in vivo*. In the first place, because of efficient excretion, metabolism, or what-not, these compounds may be almost innocuous to the living animal. Thus the lethal dose of sodium malate and sodium malonate in mice was only slightly below that of sodium chloride, while sodium maleate was scarcely more toxic. In the cases of some of the most likely amines tested (see Quastel and Wheatley, 1933), the lethal dose in mice was sufficiently high to prohibit (on grounds of expense) experiments on larger animals. On the other hand, many compounds are ex-

[4] The occasional "relapses" occurring several days after a massive dose of KCN from which the animal has apparently recovered occur under wholly different experimental conditions.

tremely toxic to the organs generally and kill the animal by their irritant or other systemic action. Thus sulphides, both organic and inorganic, caused very severe local necrosis; in any case the union between SH and haemoglobin is so firm that any effects on the nervous system would probably not be the result of inhibition by SH of intracellular oxidative ferments. We found it possible, however, to study the effects of a few compounds; the experiments are described below.

SODIUM AZIDE.

Keilin (1936) found that this compound inhibited many ferments in a manner similar to KCN. Its immediate physiological action was described in 1904 by Smith and Wolf.

Dosage.

Seventeen fasting monkeys received, on 6 (or 7) days weekly, intramuscular injections of a freshly prepared 2 p.c. solution of NaN_3 in sterile distilled water. The dose was adjusted weekly to the body-weight. The animals died or were killed after from 2 to 204 days, having received in all from 35–1,206 mg. per kilo. in from 2 to 165 doses (Table 1). With the exception of M.966 (see below), which was exceptionally resistant initially, or acquired resistance later, different monkeys varied only slightly in their tolerance to the poison.

TABLE 1.

Lesions in the brains of monkeys given repeated doses of sodium azide.

Monkey.	Time in days.	Total dose in mg. per kilo.	Range of doses in mg. per kilo.*	Number of doses.	Survived after last dose.	Lesions.					
						Necrosis of optic nerves or tracts.	Partial demyelination of optic nerves or tracts.	Necrosis in centrum semi-ovale.	Partial demyelination of centrum semi-ovale.	Necrosis in basal ganglia.	Necrosis in cerebral or cerebellar cortex.
924	2	35	15–20	2	30 mins.		No lesions in occipital lobes.				
923	6	60	10–12·5	5	40 mins.		No lesions in occipital lobes.				
932	6	59	11–12	5	40 mins.	+	0	0	0	0	±
919	11	84	5–8	11	24 hrs.†	+	0	0	0	++	0
906	11	104	8–11	10	30 mins.	0	0	0	0	0	0
921	18	102	6	17	18 hrs.	+	0	0	0	++	±
933	25	176	6–12	22	50 mins.	+	0	0	0	++	0
912	38	233	6–10	33	25 mins.	+	0	0	0	±	0
931	43	263	5–7	39	24 hrs.	+	0	0	0	++	±
910	45	233	4–12	36	18 hrs.	+	0	+	0	+	0
964	61	272	5–7	53	15 mins.	0	+	0	0	+	0
965	61	272	5–7	53	20 mins.	0	+	0	0	+	0
899	70	533	6–12	58	24 hrs.	0	±	0	0	++	±
938	99	470	5–5·5	99	1 hr.	0	±	0	0	++	±
920	126	610	5	122	40 hrs.	0	+	0	0	++	0
967	130	541	5–5·5	103	48 hrs.	0	++	0	+	0	0
966	204	1,206	5–9	165	***	0	++	0	+	0	0

* Often irregular, not more or less uniformly ascending as with KCN.

† "Relapse" of KCN type.

*** Killed in 8 mins. by very small (normally sublethal) dose of KCN.

A single large dose (20 mg. per kilo.) rendered a monkey unconscious within 6 to 8 minutes. Gradual recovery occurred during the next 8 hours, when the animal could sit up in a rather drunken manner. Next morning it appeared normal, but succumbed within a few minutes to a dose three-quarters of that tolerated on the preceding day. In other experiments, a two-day interval between successive doses did not obviate a cumulative action of the poison, or more probably of its effects (see Discussion). Animals thus treated were also more sensitive to KCN and succumbed after doses normally sublethal.

Repeated daily doses of from 10 to 12 mg. per kilo. to 7 monkeys produced at first mild, but within 3 to 4 days very severe "immediate" symptoms leading to death unless the dose were

considerably reduced. Repeated daily doses of (a) 8 mg. per kilo. [1 monkey] first produced serious symptoms on the 8th day and death after three further doses; (b) 7 mg. [1 monkey] first produced serious symptoms on the 14th day—by interpolating days with smaller or no doses the monkey was kept alive for 43 days; (c) 6 mg. [1 monkey] first produced serious symptoms on the 17th day and death after a further dose.

With daily doses of 5 mg. per kilo. [6 monkeys] mild but progressive nervous and general symptoms usually appeared after from four to six weeks. Two such animals died suddenly when two months after beginning the intoxication the daily dose was raised to 7 mg. (M.964, 965). Three others survived for from 99 to 130 days; they gradually lost condition and died without ever having shown serious nervous symptoms following immediately upon an injection. Of these five, two developed the progressive nervous manifestations described below, and two a terminal nervous condition leading to a moribund state in the course of two days. One animal (M.966) ultimately sustained daily doses of 9 mg. per kilo, but died within a few minutes of a normally sublethal dose of KCN.

Once animals showed serious "immediate" symptoms, the daily dose might have to be interrupted or reduced to as little as 4 mg. per kilo. if the monkey were to survive.

Symptoms.

After a relatively small dose the animal lay on the floor of its cage. It was restless and continually changed its position; it rose at once on the approach of the observer, but soon lay down again. This behaviour may have resulted from vertigo. Breathing was deeper and more frequent than normal; slight exertion exaggerated the hyperpnoea. On occasion retching, vomiting or evacuation of the bowel occurred. Larger doses caused dilatation of the pupils and were succeeded by drowsiness or coma, fibrillary or coarse muscular tremor, gross twitching and shaking, or tonic cramps and rigidity. Breathing became either slow, deep and gasping, or fast, jerky and irregular; typical Cheyne-Stokes respirations were not seen. Flaccidity followed rigidity. Death occurred from respiratory failure.

After repeated doses at first producing no apparent effects, except possibly deeper breathing, "immediate" symptoms when they appeared were often similar to the above. Other symptoms developed at times less closely related to a particular injection. Thus one monkey (M.921) became blind during the night. Several others, on daily doses of 5 mg. per kilo. began to suffer at any time of the day from curious "attacks" which became increasingly frequent. In the "attacks" some muscles became rigid and threw the animal into grotesque attitudes, while it performed various complicated movements. A common variant was a kind of dance in which the animal rotated in the erect position with feet, legs, tail and spine all rigidly extended, while the arms were held rigidly by the side or were extended in an Hitlerian salute. The "attacks" lasted for a few seconds or for a minute or more. In many cases the tendency to lie down after an injection disappeared with repeated doses; the animals remained sitting until they finally collapsed, as after KCN. "Immediate" symptoms appeared at a shorter interval after the provoking dose even in animals apparently completely recovered from the preceding dose. Reflex salivation when the animal was caught (see Hurst, 1940) was observed in one monkey.

As persistent symptoms, following a single large or repeated smaller doses, may be mentioned temporary or permanent blindness with dilatation of the pupils and loss of the light reflex, inco-ordination, cerebellar ataxia, paresis or rigidity especially marked in the legs, fibrillary muscular tremor, and apathy. When such symptoms developed in animals given repeated small doses, e.g. M.920, 967, they tended to progress even if no more poison were administered, and the animals were moribund within two days or so. All repeatedly poisoned animals lost weight considerably—some as much as one-third of the original. In one animal the healing of a small wound occupied an excessively long period, during which time much unhealthy granulation tissue accumulated.

One instance was observed of a monkey apparently recovered from a dose relapsing during the night and passing then into a critical nervous condition (M.919).

Pathological Examination.

During life the blood yielded no abnormal spectrum. At autopsy the lungs were bulky and distended. In cases of any standing, the brain was usually pale and the whole body appeared "bloodless". Sometimes the spleen was slightly enlarged. With the larger doses, softenings in the basal nuclei and often in the optic tracts or nerves were almost constant findings. In distribution, the lesions differed appreciably from those in poisoning with KCN; the differences are emphasized in the following description of the microscopical changes.

Lesions in the white matter. Damage to the optic nerves and tracts was more constant and severe than in poisoning with KCN. With large doses, and sometimes with repeated small doses, sudden complete necrosis occurred—most commonly perhaps in the optic nerves, but often in both nerves and tracts and on one occasion unilaterally. At first the ground tissues failed to stain normally with eosin or with van Gieson's stain; the myelin-sheaths though remaining normal in form, stained very feebly by Weil's method (Fig. 3, 4); nuclear structures other than those of the capillary endothelium gradually faded. Organization of the area took place from the periphery,

Fig. 1. Diffuse partial demyelination in the centrum semi-ovale of the monkey in chronic poisoning with KCN. Note the relative sparing of the subcortical fibres and still more of the main projection fibres. Weil stain.

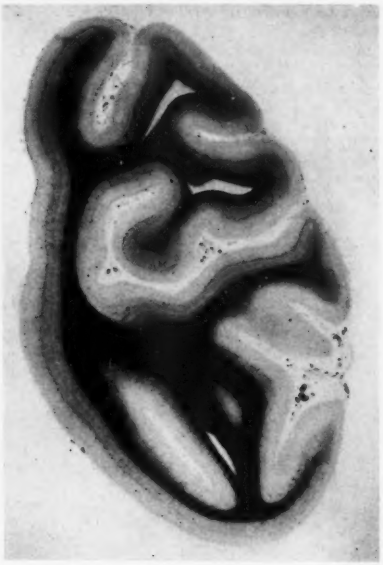

Fig. 2. Necrosis in the cerebral white matter after repeated doses of NaN_3. This appearance is common in poisoning with KCN (Hurst, 1940), rare in that with NaN_3. Weil stain.

Fig. 3. Necrosis in the optic tract of the monkey after repeated doses of NaN_3. Weil stain.

and products of myelin-disintegration appeared only as this proceeded and the area became reduced to a mass of granular corpuscles and blood vessels (cf. Hurst, 1940). In a few cases, semi-organized areas of complete necrosis were surrounded by a zone in which fresh partial demyelination was in progress. Around the most severe and more acute lesions the perivascular spaces sometimes contained a few polymorphonuclear leucocytes. In some lesions of older standing in the tracts, proliferation of large fibre-forming glial cells was far more marked than in poisoning with KCN. The central fibres of the optic nerves and tracts were invariably those affected; peripherally a wider or narrower zone of normal myelin-sheaths persisted. Necrosis did not continue beyond the sclera; in the eyeball the optic disc appeared normal, and at most minor changes were visible in the ganglionic and inner nuclear layers of the retina.

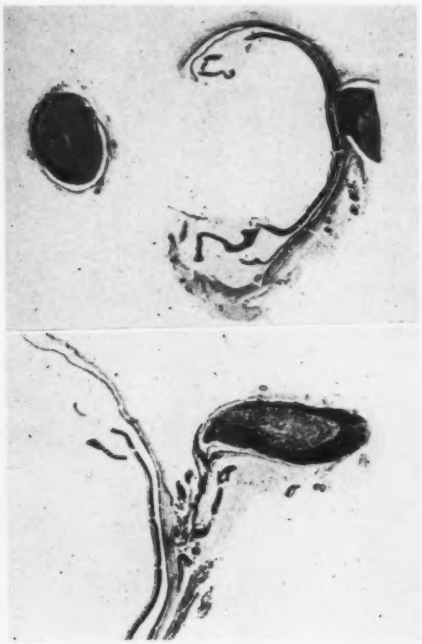

Fig. 4. Necrosis in the optic nerves of two monkeys poisoned with repeated doses of NaN_3. The necrosis stops short of the sclera. Weil stain.

As stated, sudden complete necrosis was not necessarily the concomitant of severe symptoms following a large dose of NaN_3; on occasion it occurred suddenly as the first serious manifestation in monkeys on repeated small doses. The dramatic symptomatology in some of the cases enabled us to determine precisely the minimum age of the necrotic areas. By so doing we confirmed the suspicion already expressed (Hurst, 1940), that in some instances of necrosis due to these chemical agents, the nuclear structures, etc., fade more slowly than in that due to freezing with CO_2 or to complete vascular blockage.

Most of the monkeys given repeated small doses of NaN_3 showed only partial demyelination (as previously defined—Hurst, 1940) of the central parts of the optic nerves and tracts. The axis-cylinders frequently disappeared at about the same rate as did the myelin sheaths, though in the optic tracts and nerves of M.966 the former were relatively far better preserved. Marchi-staining droplets were extremely numerous. Microglial and large neuroglial cells were abundant, oligodendroglial cells few and mostly degenerate. However we were unable to determine whether the disappearance of the last was the cause or a concomitant of the demyelination.

The earliest development of optic lesions was in a monkey receiving 5 doses (59 mg. per kilo.) of NaN_3 in the course of 6 days. Of 14 monkeys poisoned for longer periods 12 showed some degree of involvement of the optic tracts.

The white matter elsewhere seldom exhibited lesions. M.910 (Fig. 2) showed in its occipital lobes small scars very like those depicted in Fig. 2 of the earlier article (1940). One monkey showed marked gliosis (large fibre-forming glial cells) in the occipital white matter, and one similar change in parts of the cerebellar white matter. In animals given repeated small doses (e.g. M.964, 965), Marchi-staining droplets were very numerous in the deeper parts of the centrum semi-ovale; they left unaffected a narrow zone immediately beneath the cortex. In M.967 and 966 there existed clear-cut partial demyelination of the white matter of the occipital lobes. The areas affected were situated centrally in the centrum semi-ovale and not at the distal extremities of the tongues of white matter projecting into the gyri.

One monkey only (M.966) showed damage to the white matter of the upper dorsal and cervical but not of the lumbar cord. In the cervical region the degeneration was limited to the columns of Goll and had the appearance of an ascending tract degeneration. Sufficient spinal cord did not remain for us to determine the state of affairs at lower levels; there was no descending degeneration in the lumbar region.

A constant change in the white matter of animals poisoned for 11 days or more was vacuolation. The vacuoles were often extremely numerous, roughly spherical in shape, with a

flattened oligodendroglial nucleus often attached to the periphery. The contents stained very feebly with haematoxylin and with Masson's stain, they did not stain with scharlach R. With mucicarmine their pinkish colour was seldom deeper than that of the background. Possibly they represented a change in the oligodendroglia akin to the well-known mucinoid degeneration. In most control animals such vacuoles, if present, were very much fewer than after poisoning with NaN_3; they were, however, numerous in monkeys given injections of foreign protein (egg-albumin).

Lesions in the grey matter. Bilateral, though not strictly symmetrical, necrotic foci occurred in the caudate nucleus and putamen of the lenticular nucleus in 11 of 14 monkeys poisoned for 11 days or longer. They developed alike in animals suffering one or more lengthy attacks of unconsciousness and in those which on smaller doses went gradually downhill; in the latter they were usually recent and had obviously occurred during the day or two preceding death. In the nuclei under consideration partial damage as previously defined was far less common. On the other hand, the globus pallidus sometimes, and the optic thalamus, corpus subthalamicum and substantia nigra occasionally, showed areas of partial damage. At times a few apparently intact nerve-cells persisted in necrotic areas of the lenticular and caudate nuclei although all other cells, glial and nervous, had disappeared. The lesions in the grey matter differed from those in poisoning with KCN (i) in their greater frequency in the putamen and caudate nucleus, and (ii) in their less sharp margins due to a spreading-out into adjacent tissues of large fibre-forming glial cells.

Areas of necrosis in the cerebral (M.921, 931) or cerebellar (M.899, 932) cortex, associated with light polymorphonuclear infiltration in the overlying meninges, were in general similar to those in poisoning with KCN. All these monkeys had suffered long periods of unconsciousness. In M.938, smaller areas of necrosis and of partial damage occurred in the cerebral cortex although there had been no long periods of unconsciousness; many large astrocytes were present, especially in the molecular zone, and the lesions reached their maximum intensity in the occipital lobes. Only one animal (M.899) showed involvement of the cornu Ammonis.

Comment.

Necrosis or demyelination of certain areas of the white and grey matter may result from repeated administration of NaN_3; in detail and distribution the lesions differ materially from those produced by KCN. A sublethal dose of NaN_3 has much more prolonged effects than does one of KCN. Thus on the days following a large dose of the former, a monkey apparently completely recovered reacts much more severely to a second smaller dose or to one of KCN; in contrast an animal recovered from a single dose of KCN is to all intents and purposes a new animal. In consequence it is much more difficult to avoid fatal accidents in animals on repeated daily doses of NaN_3. The constancy of involvement of the central fibres of the optic nerves and tracts, often signified by clinical blindness with dilatation of the pupils and loss of the light-reflex, and the frequent absence of gross damage to the myelin in the centrum semi-ovale, contrast with the diffuse demyelination produced by KCN. Necrosis in the optic nerves and tracts may follow single or repeated large doses leading to lengthy unconsciousness, or may occur abruptly from summation of the effects of many small doses each producing few symptoms. With prolonged poisoning by small doses of the drug, partial demyelination of the nerves or tracts also may occur. Necrosis in the caudate nucleus and putamen of the lenticular nucleus is more frequent and severe than with KCN and occurs under the same conditions of dosage as do the optic lesions. The cerebral and cerebellar cortex, globus pallidus, etc., suffer less constantly and less severely. The production of large fibre-forming glial cells is distinctly more marked with NaN_3 than with KCN. The loss of weight in animals poisoned with KCN is at most 12·5 p.c. of the original; with NaN_3 it may amount to as much as 30 p.c.

HYDROXYLAMINE.

Experiences with other amines are the subject of previous comment.

According to Sollmann (1936), hydroxylamine introduced into the animal organism is decomposed into nitrite and ammonia; the former induces methaemoglobinaemia. All our animals given hydroxylamine hydrochloride yielded blood markedly abnormal in colour. My colleague, Mr. R. J. Cowan, however, failed

owing to a peculiar turbidity of the blood to obtain the absorption spectrum of true methaemoglobin, although as much as 60 p.c. of the haemoglobin was in an inactive form. Except for one monkey, these experiments were confined to sheep.

M.908 (*M. irus*) received 100 mg. hydroxylamine HCl per kilo.; within a few minutes it showed pronounced lividity and muscular twitchings. It died as the result of a second, much smaller, dose given on the following day.

In sheep, repeated doses compatible with survival for more than a few days did not cause nervous symptoms. Their effect on the blood varied with a number of factors, two of which were the spacing between the doses and the period over which the animal had been under experiment. At first many sheep were lost by repetition of a dose thought to be safe. Later Mr. Cowan estimated daily the active and inactive haemoglobin by the methods of van Slyke and Hiller (1928, 1929); he thus enabled us when necessary to interpolate days free from a dose and to keep the animals alive for considerable periods. Individual doses ranged from 25 to 50 mg. per kilo. Six sheep receiving from 150 to 3,225 mg. per kilo., in from 6 to 184 days presented no definite abnormality in the central nervous system.

NARCOTICS.

Urethane.

When first we used this substance we were unaware that Landis (1927) and others had shown that it increases greatly capillary permeability.

Six monkeys (*M. mulatta*) given repeated doses of urethane sufficiently large to induce nervous symptoms survived for from only 2 to 11 days, during which time they received from 2 to 4·5 gm. per kilo. They suffered from diarrhoea and died with generalized oedema and often with exudates into the serous cavities.

Three monkeys given much smaller doses, each insufficient to evoke visible nervous disorder, received from 4·85 to 22 gm. per kilo. in from 49 to 122 days. During this time they lost weight considerably; they died finally with oedema, etc., as did animals of the previous group. Although they showed a variety of pathological changes in the brain and viscera, none of these was relevant to the subject of the present paper.

Nembutal[5] *and Somnifane*[6].

Three monkeys (*M. irus*) received nembutal intramuscularly on Mondays, Wednesdays and Fridays of each week, and somnifane by stomach-tube on Tuesdays, Thursdays and Saturdays. The periods of unconsciousness due to nembutal were partly dependent on the amount of somnifane administered on the previous day. According to the dose of somnifane, increased at first abruptly and later very gradually—that of nembutal was kept relatively constant—the drug induced drowsiness, weakness and ataxia, or actual unconsciousness for as long as 4 hours with a subsequent period of general weakness and ataxia. Two of the three monkeys showed lesions in the white matter; these, however, were not very marked.

M.1008 died on the 21st day, three days after the last dose of somnifane. It had received 0·52 c.c. somnifane and 177 mg. nembutal per kilo. during this time. Histologically, apart from marked "vacuolation" of the white matter, there was slight increase in cellularity, partly oligodendroglial and partly microglial, in the centrum semi-ovale and corpus callosum. The glial cells of the optic tracts were enlarged, with some increase of the microglial in the central part of the tracts. This region showed slight pallor in Weil preparations, some breakdown of myelin sheaths and many Marchi-staining globules. The axons appeared normal both in the optic nerves and tracts and elsewhere. The nerve-cells universally were well-preserved.

M.1006 died 24 hours after a dose of somnifane; it had received 5·35 c.c. somnifane and 658 mg. nembutal per kilo. in the course of 61 days. "Vacuolation" was extreme in the white matter and present in a few areas of grey matter; it was most marked in the centrum semi-ovale, but present also in the cerebellum, pons, spinal cord and optic tracts. Apart from this the white matter showed "over-cellularity", which appeared to result from proliferation of both microglia and oligodendroglia; it was most marked in the centrum midway between the frontal and occipital poles. Some oligodendroglial nuclei were pyknotic and karyorrhectic, and a few glial cells possessed enlarged cell-bodies. Marchi-stained globules were very numerous. Similar changes were far less advanced in the optic nerves and tracts and in the spinal cord. The sciatic nerves were normal. The nerve-cells were mostly well-preserved, but there was one small glial-mesodermal scar in the vertical cortex. A few areas of perivascular mononuclear infiltration existed in the white matter and in the meninges.

M.1007 died within a few minutes of a dose of somnifane; it had received 15·6 c.c. somnifane and 1,715 mg. nembutal per kilo. during the preceding 141 days. Histologically, its nervous system was normal.

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[5] Sodium ethyl–(1–methyl–butyl)–barbiturate.

[6] A liquid hypnotic manufactured by Roche Products Ltd. It contains the "diethylamine salts of 0·1 gm. diethylbarbituric acid and 0·1 gm. allylisopropyl–barbituric acid".

These results suggest that, under certain conditions or in certain animals, the administration of barbiturates may lead to degeneration of the myelin. The lack of changes in the monkey longest poisoned may betoken greater resistance to poisoning, since this animal in the initial stages recovered easily from doses comparable with those killing the other two. However, it would obviously be desirable to present further observations, at the moment impossible owing to lack of monkeys.

CARBON MONOXIDE AND NITROUS OXIDE.

Though necrosis in the globus pallidus and elsewhere has attracted far more attention in the literature, the evidence that poisoning with CO may lead to demyelination, or necrosis, of the white matter in both man and the experimental animal is so definite as to exclude the need for further proof.

In recent years a number of observers have described demyelination as a sequel to poisoning with CO (Stewart, 1920; Grinker, 1925; Strecker, Taft and Willey, 1927; Meyer, 1928a; Putnam, McKenna and Morrison, 1931; Hsü and Ch'eng, 1938; Jacob, 1940; Eros, 1942). Such cases are not very common. They appear to be either those in which unconsciousness lasting for many days has followed exposure to the gas, or those in which after a shorter period of unconsciousness the patient has recovered more or less completely, only to relapse with severe nervous symptoms days or weeks later[7]. In general in these cases, demyelination has been widespread through the centrum semi-ovale but has spared the U-fibres; in places it may have been focally accentuated. In Grinker's case the axis-cylinders were relatively less affected than were the myelin-sheaths. In different cases, the internal capsules, corpus callosum, anterior commissure and cerebellar white matter have or have not been involved. The demyelination has occurred with or without the bilateral necrosis of the anterior part of the globus pallidus at one time considered pathognomonic of CO-poisoning and described by numerous writers. Less frequently areas of softening have been present in the deeper layers of the cerebral cortex or elsewhere. Grinker regarded the process in the white matter as a kind of incomplete softening; its resemblance to the lesions of diffuse sclerosis suggested a toxic origin of the latter.

Meyer (1928b) produced comparable symptoms and lesions by exposing dogs and cats to CO for one to several hours. At the onset of respiratory failure, short pauses insufficient to permit recovery of the animal from coma were needed to ensure survival. In these experiments injury to the white matter was scarcely less frequent than was necrosis of the globus pallidus; the caudate nucleus and substantia nigra occasionally suffered severely. Yant *et al.* (1934) found cystic areas in the medullary substance of the brain and focal areas of myelin-degeneration throughout the entire nervous system of dogs killed from 16 to 165 days after many hours' exposure to CO.

The pathogenesis of these lesions has been discussed at length by some of the above authors and by Hiller (1924) and Hadfield (1929). Peculiarities of vascular distribution in the globus pallidus, vascular siderosis in the globus pallidus, vascular dilatation and stasis, the Vogts' concept of "pathoclisis" and a direct action of CO on the tissues, all have been invoked to explain the development and distribution of one or other of the changes found.

Sayers and Davenport (1937, pp. 37 and seq.) summarize the clinical evidence that occasionally also the visual apparatus may be affected in poisoning with CO.

One record exists (O'Brien and Steegmann, 1938) of necrosis of the white matter of the parietal and occipital lobes following anaesthesia with N_2O; the illustration is strongly reminiscent of the more acute lesions produced by KCN.

BILATERAL NECROSIS IN THE BASAL NUCLEI.

With or without lesions in the cerebral or cerebellar cortex, severe damage to the basal nuclei results from a variety of noxious influences.

Carbon monoxide. Bilateral necrosis of the anterior part of the globus pallidus is sufficiently frequent to have been considered at one time as pathognomonic of poisoning with CO. As indicated above, Meyer (1928b) showed that sometimes in the experimental animal other nuclei may suffer more severely than the globus pallidus. Müller (1930) and Yant *et al.* (1934) also reported necrosis in the optic thalamus, etc. In a small series of cases, Hadfield (1929) found that necrosis of the globus pallidus occurred only when the vessels were the seat of deposits of iron salts; he suggested that siderosis might be a causal factor. Other hypotheses seeking to account for the frequent involvement of the globus pallidus have been mentioned in the section on poisoning with CO.

[7] The time interval in these relapses, as in those of experimental KCN-poisoning, suggests that they are not wholly (if at all) analogous to those succeeding much more transient recovery from complete anaemia of the brain (Crile and Dolley, 1908; Thorner and Lewy, 1940, etc.).

Potassium cyanide. Meyer (1933) and Hurst (1940) recorded necrosis in various nuclei in the experimental animal. The former cited two similar cases in man.

Morphine. Weimann (1926) saw the globus pallidus affected together with the cerebral cortex in a case of poisoning with pantopon. In morphine poisoning of experimental animals, especially mice, the striatum was more particularly affected.

Nitrous oxide and cyclopropane. Courville (1936) observed that the lenticular nucleus (particularly the globus pallidus—Abbott and Courville, 1938) suffers equally with the cerebral cortex and Purkinje cells of the cerebellum in poisoning with this gas. He attributed the lesions to asphyxia resulting from anoxic anoxaemia (1938), for they were similar to those produced experimentally by temporary ligation of the larger arteries of the brain. While Lowenberg, Waggoner and Zbinden (1936) took the opposite view that lesions were due to the direct toxic action of the gas on the nervous tissues, they agreed that the cortex and basal ganglia suffer much more severely than does the brain-stem and cerebellum. O'Brien and Steegmann (1938) found the cerebral cortex, caudate nucleus and putamen severely affected while the globus pallidus was only slightly shrunken. The lesions in the white matter of their case have been mentioned above. The cortex and globus pallidus were affected in Gebauer and Coleman's case of death after anaesthesia with cyclopropane (1938). This case was of the "relapsing" type mentioned above in connection with CO-poisoning. Steegmann (1939) cites cases in the literature in which comparable lesions have followed anoxia of purely mechanical nature (e.g. delayed death after hanging, ligation of the carotid artery, etc.).

Ether, carbon dioxide and chloroform. Blume and Meyer (1933) noted that severe cerebral damage results only when there has been a long but temporary failure of respiration. Of 6 cats with severe histological changes in the brain, 4 showed lesions in the globus pallidus.

Barbiturates. De Groat (1940) observed bilateral necrosis of the globus pallidus 5 days after an overdose of pentobarbital sodium. On the basis of what is known concerning the toxic effects of CO, he concluded that such necrosis may occur whenever prolonged deep asphyxia is followed by temporary survival for several days. Steegmann (1939) found severe changes in the cerebral cortex, caudate nucleus and putamen in a case dying 4 days after anaesthesia with avertin in amylene hydrate.

Cerebral trauma, etc. Seven months after a mild cerebral concussion, Rotter's patient (1929) suffered a severe psychical shock and died a fortnight later with pneumonia. Apart from changes in the cerebral and cerebellar cortex, the globus pallidus was the seat of *recent* bilateral softening. In the absence of anatomical vascular change, these lesions, which appeared clearly to be vascular in origin, could be explained only on a vasomotor basis.

Meyer (1941) described bilateral softening of the globus pallidus following cerebral trauma and fat embolism of the brain; around the pallidal lesions no emboli was found.

Bingel and Hampel (1934) and Döring (1936) describe and cite cases of lesions in the basal nuclei in cases of delayed death after hanging.

Pneumonia. Hadfield (1929) reported bilateral necrosis of the globus pallidus in a case of pneumonia in which exposure to CO could certainly be excluded.

DISCUSSION.

In this and a preceding paper (Hurst, 1940) I have considered the nervous lesions produced in one or more of three situations by relatively simple chemical compounds. The areas affected were (i) the cerebral and cerebellar cortex, (ii) the basal nuclei, and (iii) certain parts of the white matter. The observations as a whole suggest that the last two tend to suffer injury under rather similar circumstances. In any of these regions, lesions may take the form of complete necrosis of tissue over a smaller or larger area, or of "partial damage" in which only some of the constituents of the tissues are obviously injured. It is of interest that in the basal nuclei these constituents are not necessarily the nerve-cells, commonly supposed to be most vulnerable to toxic influences. The "partial damage" of the white matter (and at times of the grey matter) we have described as "demyelination", though as stated the axis-cylinders often disappeared at about the same rate as did the myelin-sheaths. Coincident destruction of axis-cylinders takes place also in several human "demyelinating" diseases, in which as a group emphasis can no longer be placed upon simple disappearance of the myelin-sheaths. Rather are these diseases characterized by major localization of the morbid process in the white matter, with relative integrity of the grey matter and especially of the nerve cells.

Because of its evanescent action, KCN is possibly most suitable for daily administration; within the limits stated in the previous paper, an animal recovered

from a number of moderate doses is in the same state of reactivity as before, while the effects of other chemicals used in this study are much more lasting. Speaking generally, with KCN cortical changes result from one or more massive doses. Necrosis of the basal nuclei and/or of more or less extensive tracts of the white matter follows repeated large doses; rarely necrosis of the centrum occurs after a single massive dose. Of the basal nuclei, the globus pallidus, caudate nucleus, putamen and substantia nigra are affected in this order of frequency. Progressive demyelination without necrosis results from oft-repeated, rather smaller doses.

Judging by the relative infrequency of nervous sequelae in human beings recovered from the immediate effects of a single inhalation of CO, the basal nuclei and centrum semi-ovale are not very commonly injured by a single exposure to this gas. However, by prolonged administration on one or a few occasions, Meyer (1928b) damaged both in a high proportion of animals. The lesions he, and Yant *et al.* (1934), produced in the white matter resembled more closely the necrosis caused by repeated large doses of KCN than the demyelination described in the present paper. In some human cases, on the other hand, the changes appear to have been more purely demyelinating in character. An important feature in human cases is the frequent history of relapse following partial or complete recovery from the effects of CO. Similar clinical relapses occur experimentally with KCN and NaN_3, though we could not correlate them closely with a particular kind of nervous lesion. With McCall (1940) we believe that existing hypotheses concerning the genesis of the lesions in man do not account satisfactorily for this phenomenon (see also footnote on page 307). We have referred also to anaesthesia with N_2O, etc., as preceding necrosis in the basal ganglia.

A few large or many smaller doses of NaN_3 may cause necrosis or demyelination, as the case may be, in the optic nerves and tracts; only rarely are marked changes seen in the centrum semi-ovale. In the basal nuclei, affected very frequently, the caudate nucleus and putamen suffer in preference to the globus pallidus. Cortical lesions are relatively less severe and frequent; they occur both with large and with small doses. In fact, necrotic lesions in all three situations may develop abruptly in animals given repeated small doses of NaN_3, each insufficient to evoke marked nervous symptoms. The sudden blindness, etc., occuring under these circumstances present an apparent analogy to the equally sudden visual or other disturbance heralding the onset or marking a recrudescence of disseminated sclerosis in man. Here the abrupt symptomatology has suggested an infective process rather than the summation of a number of chemical disturbances. Consideration of the response to daily or irregular doses of NaN_3, of constant or variable amount, shows that these sudden marked effects cannot possibly be explained on the basis of mere accumulation of the poison in the system, unless the animals lose progressively their ability to excrete or otherwise dispose of it and at different times excretion is wholly erratic. It seems more probable that repeated small insults to the nervous tissues bring these to a state in which a further small dose of poison, one normally tolerated, produces the most serious consequences. This hypothesis is in keeping with the analogous facts we have described in poisoning with KCN; although rapidly detoxicated and not cumulative in the body in the ordinary pharmacological sense, KCN administered daily may suddenly produce necrosis involving almost the whole of the centrum semi-ovale with doses previously tolerated many times.

In determining the mechanism by which these lesions are produced and in explaining their distribution within the nervous system, the hypotheses advanced in the case of CO-poisoning in man do not prove very helpful. Vogt's theory of "pathoclisis", or selective vulnerability of a particular area of the brain, does not easily explain the diversity of sites which may be affected with either KCN, CO or NaN_3. In the lesions caused by KCN and NaN_3 there is no evidence of the

vascular dilatation, stasis, etc., alleged to play an important part in those due to CO. It is equally unconvincing to attribute these lesions to vascular spasm. The areas of necrosis resulting from vascular blockage differ materially in distribution and outline from those under discussion (Hurst, 1940). The differences in the sites affected according to whether KCN is administered in maximal or submaximal doses, or KCN is replaced by NaN_3, cannot easily be reconciled with a theory of pathogenesis which places major emphasis upon a purely vascular factor.

Our experiments to show whether or not after a dose of KCN the brain is more accessible to toxic substances possibly present in the blood gave negative results; only if animals were continuously seriously ill or moribund did methylene blue, in abnormal amounts, penetrate and remain fixed (and unreduced) for considerable periods in the nervous tissues. On the other hand Siengalewicz (1924) reported that CO increased the permeability of the choroid plexuses and meninges.

In view of the known action of KCN and NaN_3 on the oxidative ferments of cells, it seemed reasonable to enquire whether the demyelination produced by the poisons could be explained on this basis. Wyndham's work (1941) affords no support in this direction. Though *reversible* inhibition may account for the immediate symptoms of poisoning, *irreversible* inhibition of these ferments was not brought about with concentrations of KCN far greater than any likely to obtain in our experiments, and no decrease in the activity of oxidative ferments was observed in the white matter of monkeys long poisoned with KCN. In the course of further investigations, Mr. Wyndham[8] (personal communication) estimated the polydiaminophosphatide, cerebroside, polydiaminophosphatase and cerebrosidase in the grey and white matter of monkeys and sheep. Of the former, 4 had received NaN_3 and 3 KCN for longer or shorter periods before death. On the latter, 5 had received KCN and 3 hydroxylamine, while two were untreated. The variable results led him to believe that "it is not possible to produce any demonstrable alteration in the proportions of these brain-constituents by giving to monkeys and sheep daily injections of the three substances used. Only in the case of the phosphatide was there found consistently more of the substance in the white matter than in the grey; this is the only generalization to be made from the work apart from confirmation of the observations of earlier workers that sulphydryl compounds have inhibiting action upon polydiaminophosphatase and activating action upon cerebrosidases in both grey and white matter of the brain."

Other observations also suggest that the chemical agents causing demyelination do not operate by direct action on the oxidative enzymes of cells. Although biochemically NaN_3 has an action similar to that of KCN, its biological action differs materially and only presents new problems regarding the distribution of lesions within the nervous system. In the case of CO, it has been shown that the gas has an affinity for Hb many times that of the respiratory ferment of cells; Haggard (1922) found that nerve-cells of the chick may live and grow normally in the presence of as much as 80 p.c. CO (and 20 p.c. O_2). Yant *et al.* have shown that, at any rate in short experiments, the nervous lesions of dogs asphyxiated by atmospheres merely deficient in O_2 are quite similar to those after equivalent exposure to CO. Similarly with nitrous oxide, Courville (1938) attributed the lesions to anoxic anoxaemia rather than to any specific toxic action of the gas. De Groat (1940) concluded that necrosis of the globus pallidus might occur whenever prolonged deep asphyxia was followed by temporary survival for a few days. It is true that the last three observations concern the grey rather than the white matter, and that demyelination, whether produced by CO, KCN or NaN_3, may be a separate phenomenon. Nevertheless demyelination must be mediated by enzymatic processes, and must ultimately be explained on a biochemical basis.

[8] Mr. Wyndham left the Staff of this Institute to undertake work of national importance before the present studies were completed.

The question at present under consideration is whether it arises directly from a specific poisoning of a particular enzyme-system, in which case knowledge of the exact system affected might provide a clue to the origin of demyelination in man, or whether during a period of anoxia (histotoxic or otherwise) the cellular enzyme-systems as a whole are thrown out of balance, which would suggest that demyelination is merely a type of reaction on the part of the nervous system and probably of multiple causation.

However this may be, the whole trend of our observations and those of others is to show a continuous series of lesions with cortical necrosis at the one extreme and demyelination of the white (or grey) matter at the other. In our experiments the most severe anoxia led to major damage to the cerebral or cerebellar cortex, less severe anoxia to necrosis of the basal nuclei, while necrosis in the basal nuclei or white matter and demyelination in the white matter resulted from repeated smaller insults each insufficient seriously to damage the cortex. If we could assume a poor recuperative capacity on the part of the white matter, possibly connected with its known deficiency in oxidases or the comparative poverty of its capillary anastomoses, a number of relatively minor insults each insufficient permanently to damage the grey matter might be summated into either necrosis or demyelination according to their intensity.

SUMMARY.

Under the conditions described in the present paper, repeated administration of KCN to monkeys led to progressive demyelination, without necrosis, of the cerebral white matter. In distribution the lesions resembled closely those of Schilder's disease. In two monkeys the encephalopathy did not progress when poisoning was discontinued; in four animals its presence did not apparently favour the development of post-vaccinal encephalitis. Repeated massive doses of KCN followed by resuscitation led in two monkeys to widespread necrosis of the cerebral and cerebellar cortex rather than to damage of the basal nuclei or white matter. Although methylene blue given intravenously may penetrate and remain fixed and unreduced for days in the brains of monkeys seriously ill after large doses of KCN, we could not demonstrate an increased permeability or affinity of the brain for the dye, or a lack of its reduction, in poisonings short of those fatal. In sheep and rabbits the response to injections of KCN is more erratic than in the monkey.

The symptomatology of poisoning with NaN_3 in the monkey is described. The effect of a single dose is more lasting than that of KCN and renders the animal more susceptible to subsequent administration of KCN or NaN_3. Repeated administration frequently produces necrosis or demyelination in the optic nerves and tracts and necrosis in the caudate nucleus and putamen of the lenticular nucleus. Necrosis may follow a single or repeated large doses with lengthy unconsciousness or may occur abruptly from summation of many small doses each producing few symptoms. Lesions in other parts of the grey and white matter are much less frequent and severe.

In sheep repeated administration of hydroxylamine hydrochloride in amounts compatible with survival for some weeks or months did not lead to demyelination within periods up to 184 days.

In two monkeys poisoned with barbiturates early changes were present in the white matter. Confirmatory evidence of the action of these compounds is desirable.

A survey of the literature shows that, in man or animals, demyelination in the cerebral white matter or necrosis of the basal nuclei (or both) may follow a single or a few prolonged exposures to CO. Some other causes of necrosis in the basal ganglia are listed.

Subject to revision, the view is expressed that the experimental demyelination described in this paper is a function of repeated periods of anoxia, each insufficient to produce lesions in the grey matter.

Acknowledgments. I am indebted to Dr. C. S. Swan, Miss Barbara Cooke and Mr. P. Melvin for assistance in these experiments, and to various colleagues throughout Australia for much criticism and advice.

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